Histol Histopath (1989) 4: 167-171

Histology and Histopathology

Effect of dicarboxylic (C_6 and C_9) acids on a human squamous carcinoma cell line in culture

H.C. Pätzold[1], A.S. Breathnach[1], E.J. Robins[1], M.E. Daridan[1], Y.P. Bhasin[1], L.B. Ethridge[1], M. Nazzaro-Porro[2], S. Passi [2] and M. Picardo[2]

[1]Department of Anatomy and Cell Biology, St. Mary's Hospital, Medical School, London, U.K. and [2]Istituto Dermatologico S. Gallicano, S. Gallicano, Rome, Italy

Summary. In tissue culture, azelaic acid (C_9) has been shown to have an anti-proliferative and cytotoxic effect on human and murine malignant melanocytes, with inhibition of mitochondrial oxido-reductase enzymes and DNA synthesis, and damage to mitochondria. Recent reports of effects on differentiation of normal keratocytes have led to the present study of its effects on a squamous carcinoma cell line.

Cells were exposed to single doses of disodium salts of azelaic ($C_9$2Na) and adipic ($C_6$2Na) acids at concentrations of 10^{-2}M and 5×10^{-2}M for 48 hrs. Only $C_9$2Na at 5×10^{-2}M for 4 hrs., and longer, significantly affected proliferation, and the cells exhibited massive swelling of mitochondria with loss of cristae.

The results further confirm the probable value of azelaic acid as a general anti-tumoral agent rather than a specifically melanocytotoxic one. They could justify clinical studies on the effect of topical azelaic acid therapy on squamous cell carcinoma *in vivo*.

Key words: Dicarboxylic acid, Squamous carcinoma, Culture

Introduction

Azelaic acid, topically applied, has been shown to be effective clinically against hyperactive and abnormal melanocytes in melasma (Nazarro-Porro et al., 1978) and lentigo maligna (Nazarro-Porro et al., 1979; 1982), and to cause regression of lesions of primary cutaneous melanoma (Nazarro-Porro et al., 1980). In all these conditions so treated, there is no residual hypochromia nor apparent damage to normal epidermal keratocytes. In cell culture, at concentrations greater than 10^{-3}M, azelaic acid has an anti-proliferative and anti-viability effect of malignant melanocytes of human and murine origin with inhibition of DNA synthesis and of mitochondrial oxido-reductase enzymes, and physical damage to mitochondria (Robins et al., 1985). These effects are dose and time dependent, reversible, and not due to perturbation of H-ion concentration, or osmolarity (Robins et al., 1985; Leibl et al., 1985). Similar effects have been demonstrated for other tumoural cell lines (Leibl et al., 1985; Picardo et al., 1985) but in general, normal cells have been reported to show no effect (Leibl et al., 1985; Picardo et al., 1985; Geier et al., 1986; Hu et al., 1986). The effects on tumoural cells are related to the chain-length of the diacid. Thus, the C_6 diacid has no effect on proliferation or morphology at concentrations less than 10^{-1}M, and little effect above this, while the C_9 and C_{12} diacids are significantly effective at 10^{-2}M (Robins et al., 1985; Breathnach et al., 1986; 1987).

Recently, there have been reports of reversible dose-dependent effects of azelaic acid on proliferation, DNA synthesis, and total protein synthesis of neo-natal mouse (Detmar et al., 1986) and human keratocytes (Detmar et al., 1987) in culture, and of ultra-structural damage to normal keratocytes in vivo following application of topical azelaic acid (Detmar et al., 1987), though this latter observation contradicts a previous report (Nazarro-Porro et al., 1979). It has been suggested that azelaic acid may act as a modulator of keratocyte differentation by interfering with specific keratin precursors (Gollnick et al., 1986), and we have individual experience (unpublished) of a beneficial effect on solar keratosis and Bowen's disease. Taken overall, these latter reports might justify investigation of a possible beneficial effect of topical azelaic acid on squamous cell carcinoma *in vivo*, and, as a preliminary to this, we report here on its effect on a squamous cell line in cell culture.

Materials and methods

Cells of a squamous cell carcinoma line SCC 25, derived from epidermal carcinoma line A431 (Giard et

Offprint requests to: Professor A.S. Breathnach, UMDS Division of Anatomy, St. Thomas's Campus, Lambeth Palace Road, London, SE1 7EH, England

al., 1973) and kindly donated by Dr. A. Rothman, London Hospital were seeded in 35 mm plastic Petri dishes at an initial inoculum of about 4.8×10^5 cells/Petri dish. The cells were grown in 3 ml of Dulbecco's modified Eagle's medium supplemented with 4500 mg/l glucose, 10% fetal calf serum, 2 mM glutamine (all GIBCO), 100 IU/ml penicillin (GLAXO) and 100 µg/ml streptomycin (EVANS) at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 hours medium was changed and appropriate drug concentrations were added.

Cell counts

Adipic acid ($C_6$2Na), and azelaic acid ($C_9$2Na) were added to the medium as disodium salts at a final concentration of 10 and 50 mMol (1×10^{-2}M and 5×10^{-2}M). After 2, 6, 24 (day 1) and 48 (day 2) hours, the medium was removed, and the cells were washed in phosphate-buffered saline (PBS, OXOID), trypsinized and counted by coulter counter. To investigate the reversibility of the drug effect after exposure, the medium of parallel experimental cell cultures was replaced by medium without drugs after 48 hours, and the cells were incubated for another 24 (day 3) to 96 (day 6) hours before counting. Three runs of the above procedure were carried out for each diacid at the various times and concentrations, and two counts per dish were made for each run. There were appropriate counting controls. Results were analysed statistically and experimental data were expressed as percentages of the equivalent control values (Fig. 1).

Electron microscopy

Confluent cultures of cells were exposed to each of the diacid salts at the same concentrations as above, for 1, 4 and 24 hours. After incubation the cells were washed in PBS and fixed for 5 minutes at room temperature in 2.5% glutaraldehyde in 0.2M cacodylate buffer, post-fixed in 2% aqueous osmium tetroxide and dehydrated in graded ethanols. At 70% ethanol they were scraped off the Petri dish and spun down to a pellet. After complete dehydration, the pellet was embedded in Araldite resin, and thin sections, stained with uranyl acetate and lead citrate, were examined by transmission electron microscopy.

Results

Cell Counts

As can be seen from Fig. 1, neither $C_9$2Na, at 10^{-2}M nor $C_6$2Na at 10^{-2}M or 5×10^{-2}M, had an effect on cell growth. With $C_9$2Na at 5×10^{-2}M, however, the number of cells fell significantly within 24 hrs. of exposure, and dropped to 5.2% of control value after 48 hours. After replacement of medium with new medium without diacids, cells previously exposed to $C_9$2Na at 5×10^{-2}M did not recover, and almost died off completely (to under 1% of control), over the period tested (24-96 hrs.). All other groups of cells continued to grow at the same rate as the controls.

Electron Microscopy

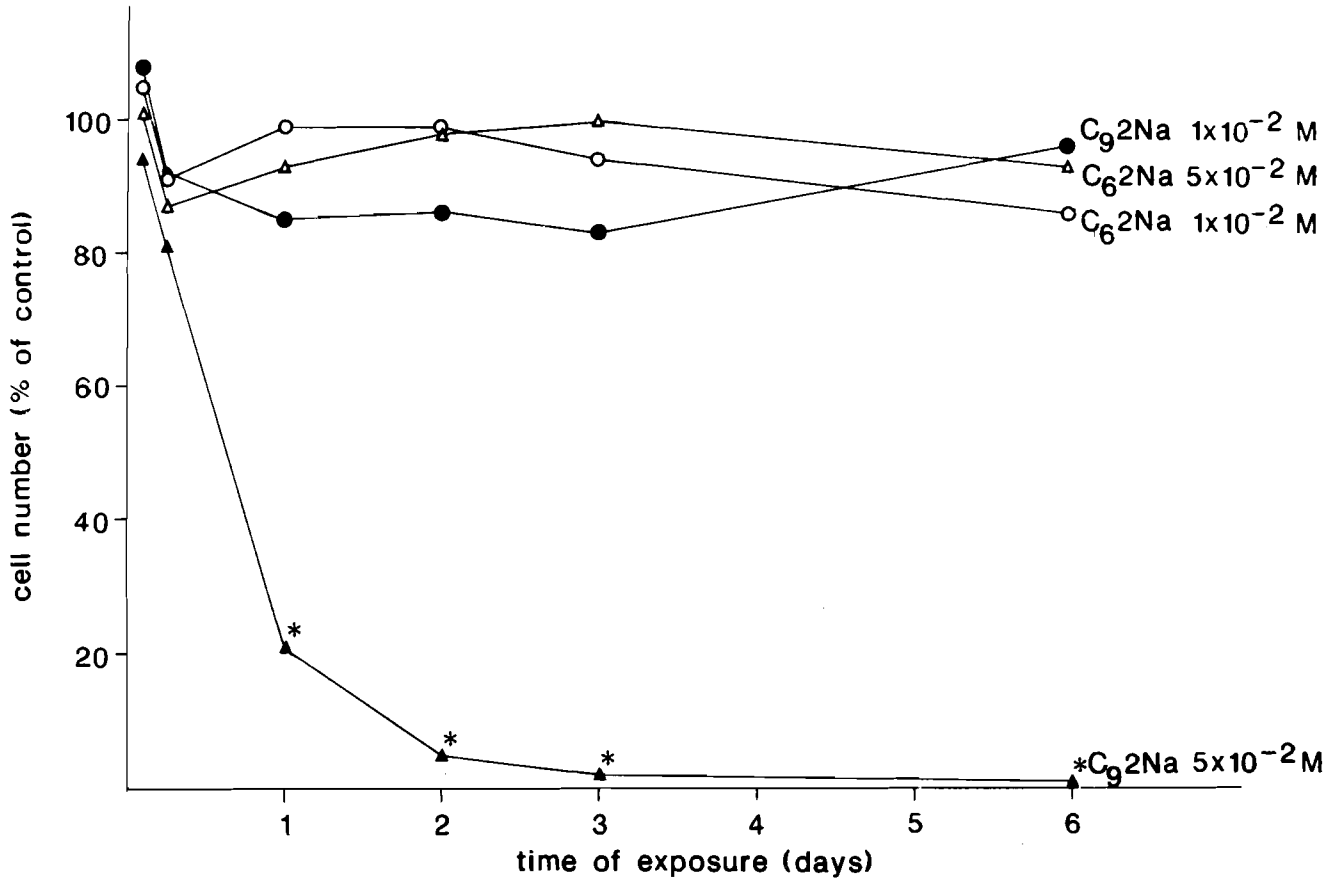
A typical cell of a control culture is illustrated in Fig. 2. Cells of cultures exposed to both diacids (C_6 and C_9) at 10^{-2}M for up to 4 hours were indistinguishable from controls. With C_9 at 10^{-2}M for 24 hrs, mitochondria were swollen with disruption of cristae and loss of internal substance, the latter feature manifested by lower electron density (Fig. 3). With C_6 at 10^{-2}M for 24 hrs, loss of cristae and internal substance was not evident (Fig. 4). At 5×10^{-2}M for 1 hr, cells exposed to C_9 showed a degree of mitochondrial swelling, but mitochondria of cells exposed to C_6 appeared no different to those of controls. At 5×10^{-2}M for 4 hrs, cells exposed to C_9 again showed massive disruption of mitochondria (Fig. 5) but with C_6 the effect was minimal, and even at 5×10^{-2}M for 24 hrs (Fig. 6) the mitochondria were demonstrably less affected than with C_9 at the same concentration for 4 hrs. (Fig. 5).

Discussion

This study has shown that, in culture, azelaic acid (C_9) has the same time and dose-dependent anti-proliferative and anti-mitochondrial effects on squamous carcinoma cells as on cells of other tumoural lines. It has further confirmed, with an additional cell line, the significantly less effect on proliferation and morphology of C_6 as compared with C_9. C_6 acts as an additional control for the effect of C_9, and underlines its specificity. The results are in line with recent reports, mentioned in the Introduction, of effects on normal keratocytes in culture, and could be seen to justify direct controlled trials *in vivo* on the effect of topical azelaic acid on selected lesions involving hyper-proliferative and malignant keratocytes. They underline again the conclusions that azelaic acid is not specifically anti-melanocytic, and, that because of its mode of action against essential enzymatic activities common to all cells (Passi et al., 1984) and DNA synthesis (Leibl et al., 1985), it has potential as a general anti-tumoural agent (Breathnach et al., 1984).

Acknowledgements. This work was supported by grants from Schering AG, Berlin, The Wellcome Trust, and by grant No. 86.0069.144, Project Oncologia, CNR, Italy.

Fig 1. Effect of $C_6$2Na and $C_9$2Na on squamous carcinoma cells in culture. Cell counts are expressed as a percentage of controls. After day 2 the medium containing diacids was replaced by medium without diacids. o-o $C_6$2Na 1×10^{-2}M, △-△ $C_6$2Na 5×10^{-2}M, •-• $C_9$2Na 1×10^{-2}M, ▲-▲ $C_9$2Na 5×10^{-2}M. *$p < 0.005$.



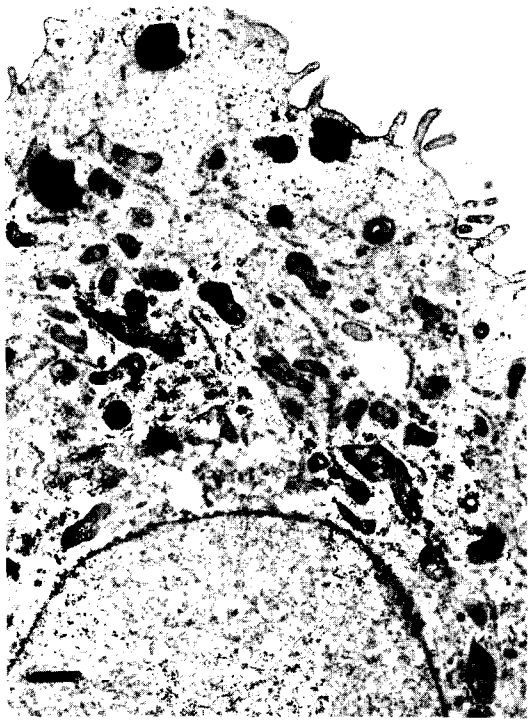


Fig. 2. Control squamous carcinoma cell. × 7,500. Bar = 1 μm.

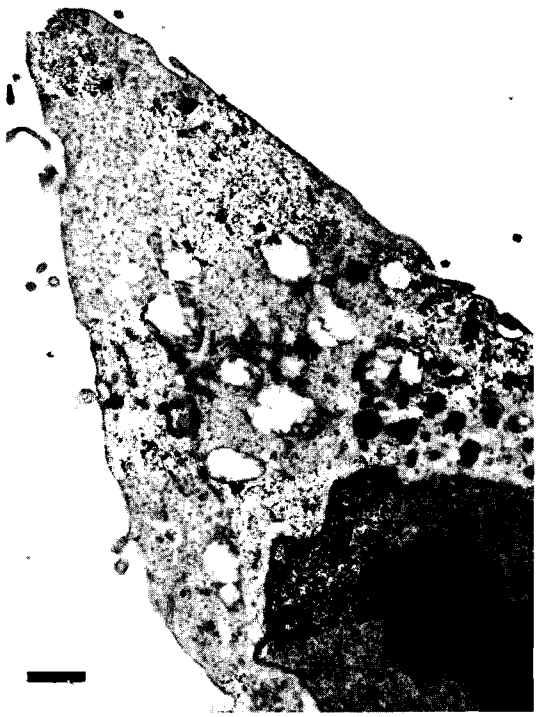


Fig. 3. Squamous carcinoma cell exposed to C_9 2Na at 10^{-2}M for 24 hrs. Note swelling and disruption of mitochondria. × 7,500. Bar = 1 μm.

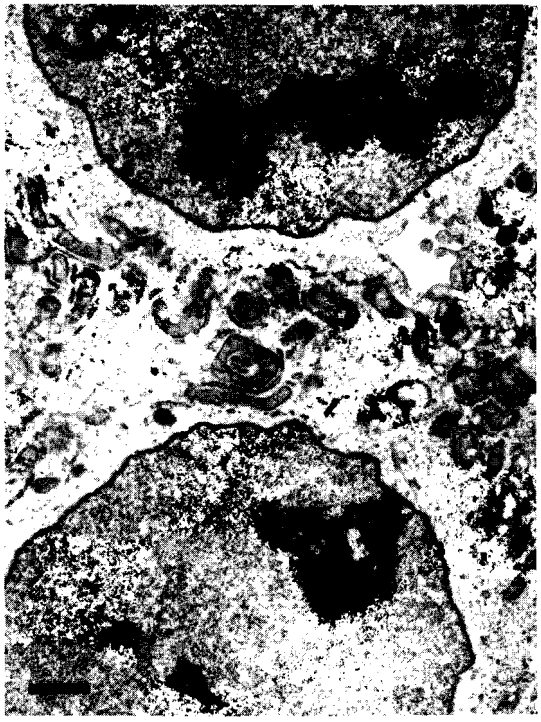


Fig. 4. Squamous carcinoma cell exposed to $C_6$2Na at 10^{-2}M for 24 hrs. Mitochondria are practically unaffected compared with control (Fig. 2). Compare also with Fig. 3. × 7,500. Bar = 1 μm.

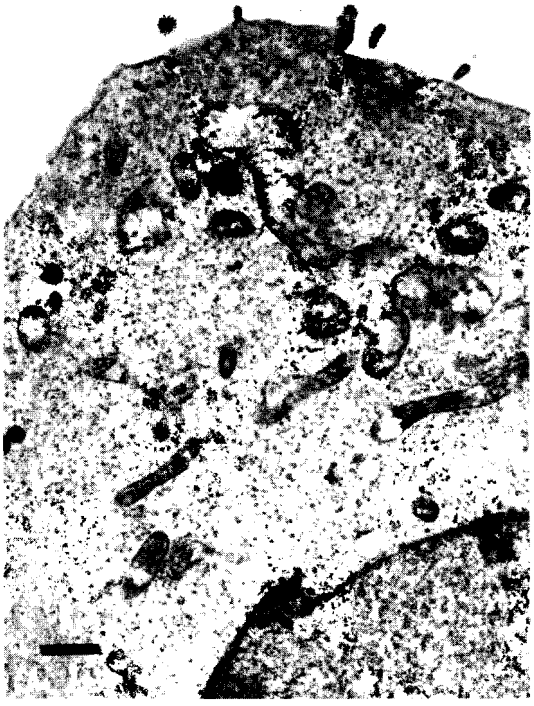


Fig. 6. Squamous carcinoma cell exposed to $C_6$2Na at 5 × 10^{-2}M for 24 hrs. There is some swelling of mitochondria but not nearly the same degree of disruption as with $C_9$2Na at lower concentration (Fig. 3), or for shorter period at the same concentration (Fig. 5). × 7,500. Bar = 1 μm.

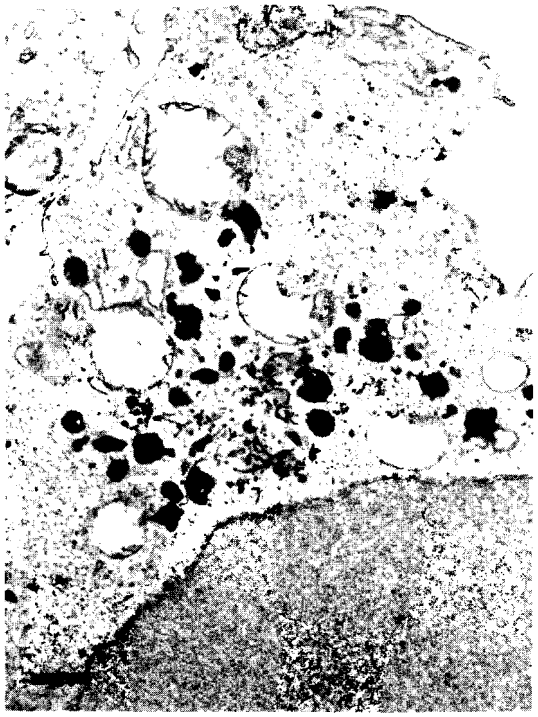


Fig. 5. Squamous carcinoma cell exposed to $C_9$2Na at 5 × 10^{-2}M for 4 hrs. Note massive swelling and disruption of mitochondria. × 7,500. Bar = 1 μm.

References

Breathnach A.S., Nazarro-Porro M. and Passi S. (1984). Azelaic acid. Brit. J. Dermatol. 11, 115-120.

Breathnach A.S., Robins E.J., Bhasin Y., Ethridge L., Nazarro-Porro M. and Passi S. (1986). Observations on cell kinetics and viability of a human melanoma cell line exposed to dicarboxylic acids in tissue culture. Histol. Histopath. 1, 235-240.

Breathnach A.S., Robins E.J., Bhasin Y., Ethridge L., Nazarro-Porro M., Passi S. and Picardo M. (1987). Scanning electron microscopy of human and murine melanoma cells exposed to medium chain-length (C_6-C_{12}) dicarboxylic acids in tissue culture. Histol. Histopath. 2, 291-297.

Detmar M., Miller R., Stadler R. and Orfanos C.E. (1986). Dicarbonsauren hemmen das Wachstum von Keratinozytem in vitro. Der Hautarzt 37, 625-627.

Detmar M., Muller K., Gassmuller H., Stadler R. and Orfanos C. (1987). Effects of azelaic acid on protein synthesis and proliferation of cultured human keratocytes. Proc. 17th World Congress of Dermatology, Berlin, Pt. 2, 456.

Geier G., Hauschild T., Bauer R. and Kreysel W. (1986). Der Einfluss von Azelainsaure auf das Wachstum von Melanomzellkulturen in Vergleich zu Fibroblastenkulturen. Der Hautarzt. 37, 146-148.

Giard D.J., Aaronson S.A., Todaro D.J., Arnstein P., Kersey J.H., Dosik H. and Parks W.P. (1973). In vitro cultivation of human

tumours. Establishment of cell lines derived from a series of solid tumours. J. Nat. Can. Inst. 51, 1417.

Gollnick H., Mayer-da-Silva A. and Orfanos C.E. (1986). Effects of azelaic acid on filagrin, other cytokeratins, and on the ultrastructure of human keratocytes in vivo. XIV Jahrestagung der Arbeitsgemeinschaft Dermatologische Forschung, Freiburg, 90.

Hu F., Mah K. and Teramura J.D. (1986). Effect of dicarboxylic acids on normal and malignant melanocytes in culture. Brit. J. Dermatol. 114, 17-26.

Leibl H., Stingl G., Pehamberger H., Korschen H., Konrad K. and Wolff K. (1985). Inhibition of DNA synthesis of melanoma cells by azelaic acid. J. Invest. Dermatol. 85. 417-422.

Nazzaro-Porro M. and Passi S. (1978). Effetto degli acid dicarbossilici in alcune dermatosi pigmentarie. G. It I. Dermatol. 113, 401-404.

Nazzaro-Porro M., Passi S., Balus L., Breathnach A.S., Martin B. and Morpurgo G. (1979). Effect of dicarboxylic acids on lentigo maligna. J. Invest. Dermatol. 73, 296-305.

Nazzarro-Porro M., Passi S., Zina G., Bernengo A., Breathnach A.S., Gallagher S. and Morpurgo G. (1980). Effect of azelaic acid on human malignant melanoma. Lancet ii, 1109-1111.

Nazzaro-Porro M., Passi S., Breathnach A.S. and Zina G. (1980). 10 years observations on the effect of azelaic acid on lentigo maligna. J. Invest. Dermatol. 78, 331.

Passi S., Picardo M., Nazzaro-Porro M., Breathnach A.S., Confaloni A.M. and Serlupi-Crescenzi G. (1984). Antimitochondrial effect of saturated medium chain length (C_8 to C_{13}) dicarboxylic acids. Biochem. Pharmacol. 32, 103-108.

Picardo M., Passi S., Sirianni M.C., Fiorilli M., Russo G.D., Cortesi E., Barile G., Breathnach A.S. and Nazzaro-Porro M. (1985). Activity of azelaic acid on cultures of lymphoma- and leukaemia-derived cell lines, normal resting and stimulated lymphocytes, and 3T3 fibroblasts. Biochem. Pharmacol. 34, 1653-1658.

Robins E.J., Breathnach A.S., Ward B.J., Bhasin Y., Ethridge L., Nazzaro-Porro M. and Passi S. (1985). Effect of dicarboxylic acids on Harding-Passey and Cloudman S91 melanoma cells in tissue culture. J. Invest. Dermatol. 85, 216-221.

Accepted October 21, 1988