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Studies on the teratogen pharmacophore of valproic acid analogues: evidence of interactions at a hydrophobic centre

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Abstract

Propyl-4-yn-valproic acid (2-propyl-4-pentynoic acid), an analogue of valproic acid with a triple bond in one alkyl side chain, potently induces exencephaly in mice. Given that propyl-4-yn-valproic acid is a branched chain carboxylic acid, we synthesized a series of analogues with *n*-alkyl side chains of increasing length and correlated their potential to induce neural tube defects and to inhibit proliferation and induce differentiation in cells of neural origin, the latter being crucial to the orderly structuring of the embryo. All analogues significantly increased the incidence of neural tube defects in the embryos of dams exposed to a single dose of 1.25 mmol/kg on day 8 of gestation. This effect occurred in a dose-dependent manner and the rate of exencephaly increased with the progressive increase in *n*-alkyl side chain length. Moreover, increasing chain length resulted in a dose-dependent inhibition of C6 glioma proliferation rate over a concentration range of 0–3 mM and this was independent of the cell type employed and mode of estimating proliferative rate. The antiproliferative action of these analogues was associated with profound shape change in neuro-2A neuroblastoma involving extensive neuritogenesis and an associated increase in neural cell adhesion molecule (NCAM) prevalence at points of cell–cell contact, the latter exhibiting a dose-dependent increase when the *n*-alkyl chain was extended to five carbon units. These results suggest an interaction with a specific site in which the *n*-alkyl side is proposed to serve as an 'anchor' within a hydrophobic pocket to facilitate the ionic and/or H-bonding of the carboxylic acid and high electron density of the carbon–carbon triple bond.

Keywords: Neural tube defect; Embryo, mouse; Proliferative rate; Differentiation; NCAM (neural cell adhesion molecule)

1. Introduction

Embryos of mice exposed to high doses of valproic acid reproducibly show exencephaly—a neural tube defect closely related to the malformation seen in humans (Kao et al., 1981; Nau et al., 1981a,b; Turner et al., 1990). Investigations of various metabolites have shown that the teratogenicity is caused by valproic acid itself and not by one of its metabolites (Nau, 1986a). Although these studies have failed to reveal a molecular or cellular rationale for the teratogenic activity of valproic acid, in vitro studies have demonstrated this agent to potently inhibit neural cell proliferation rate at concentrations within twice its therapeutic plasma level (Regan, 1985; Cinatl et al., 1996). This antiproliferative action occurs at a defined restriction point in the G1 phase at which cells assume a differentiated phenotype as judged by altered morphology, gene expression and cell-substratum adhesivity (Martin et al., 1988; Martin and Regan, 1988, 1991; Berezin et al., 1996). This antiproliferative effect may perturb signal transduction events which, in part, involve suppression of transient glycoprotein sialylation required for passage through this phase of the cell cycle (Maguire and Regan, 1991; Chen et al., 1996; Bacon et al., 1997; O'Brien and Regan, 1998). The altered glycoconjugates appear to include those medi-

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ating cell adhesion molecule (CAM) functions which play a fundamental role in neurodevelopment (Bacon et al., 1998). As valproic acid sequesters specifically into the neuroepithelium (Dencker et al., 1990), the consequence of this specific antiproliferative action is likely to result in growth imbalances arising from alterations in differential cell proliferation rates between endodermal and neuroepithelial cell populations, as have been associated with the increased incidence of spina bifida observed in curly tail (ct) mutant mouse homozygotes (Copp et al., 1988).

The induction of neural tube defects by valproic acid in vivo is reliant on strict structure–activity requirements and similar structural requirements are necessary to concomitantly inhibit proliferation and induce differentiation in vitro (Nau and Löscher, 1986; Nau and Hendrickx, 1987; Nau et al., 1991; Regan et al., 1991; Elmazar et al., 1993; Courage-Maguire et al., 1997). These structure–activity relationships require that the C2 atom bear a carboxylic group, one hydrogen atom and two straight chain alkyl groups; primary amides and unbranched analogues, or those bearing an additional methyl group (Bojic et al., 1996) or cycloalkanoic acids are not teratogenic. In addition, the sp^3-configuration at carbon atom C2 is essential as analogues in which C2 is sp^2-hybridized do not induce neural tube defects in animal models. More recently, additional valproic acid analogues with side chain modifications have been synthesized and evaluated for teratogenicity in the mouse. Among them 2-propyl-4-pentynoic acid (propyl-4-yn-valproic acid) proved to be approximately twice as potent a teratogen as valproic acid itself (Hauck and Nau, 1989; Hauck and Nau, 1992; Hauck et al., 1992). As propyl-4-yn-valproic acid contains a chiral center at carbon atom C2, the enantiomers of this analogue have been tested for their potential to induce exencephaly in mice (Hauck et al., 1992). The *R*-enantiomer was found to be ineffective whereas the *S*-enantiomer showed high teratogenic potency. Moreover, asymmetric analogue-induced change in cell shape, motility and neuritogenesis is specific to the *S*-enantiomer (Berezin et al., 1996; Bacon et al., 1998). These increased effects can be attributed to the presence of the triple bond as all other structures in the molecule are equivalent to valproic acid. Moreover, it has been demonstrated that pharmacokinetic differences are not responsible for the increased teratogenicity of propyl-4-yn-valproic acid (Hauck et al., 1992) and that the teratogenic potential of valproic acid analogues is unrelated to lipophilicity (Bojic et al., 1996).

The foregoing studies have provided a basis to search for the fundamental structural elements (pharmacophore) responsible for the teratogenic activity of valproic acid-related compounds. The studies with propyl-4-yn-valproic acid provide evidence to suggest the existence of a stereospecific site within the embryo capable of accommodating the ionic and/or H-bonding of the carboxylic acid and high electron density of the carbon–carbon triple bond. The role of the *n*-alkyl side chain remains to be established but may serve to 'anchor' the molecule in a hydrophobic pocket as any additional methyl-branching eliminates it's teratogenicity (Bojic et al., 1996). To provide further evidence for the existence of this 'hydrophobic pocket' we designed a homologous series of molecules based on the propyl-4-yn-valproic acid structure which contains *n*-alkyl chains of increasing length and determined their pharmacological properties in vivo with respect to their teratogenic potential, and in vitro with respect to their anti-proliferative and pro-differentiative properties.

2. Materials and methods

2.1. Synthesis of analogues

2-Propyl-4-pentynoic (propyl-4-yn-valproic acid) and 2-(2-propynyl)-hexanoic acid (butyl-4-yn-valproic acid) were synthesised by procedures described previously (Hauck and Nau, 1992). Synthesis of 2-(2-propynyl)-heptanoic acid (pentyl-4-yn-valproic acid), 2-(2-propynyl)-octanoic acid (hexyl-4-yn-valproic acid), and 2-(2-propynyl)-nonanoic acid (heptyl-4-yn-valproic acid) was carried out using a similar malonic ester sequence. For these higher homologues of the series it was more convenient to use unbranched carboxylic ester anions as these are easily alkylated with propargylic bromide. The resulting 2-(2-propynyl)-alkanoic acid methyl esters are distillable thereby providing the possibility to purify them from unreacted unbranched esters. Saponification of the ester yielded the free acids which were further purified by distillation and chromatographic procedures.

2.1.1. Alkylation

At 4°C a solution containing 0.1 mol lithium diisopropyl amide (LDA) in 200 ml dry tetrahydrofurane (THF) was prepared within 15 min, cooled to −70°C and 0.1 mol alkanoic acid methyl ester in a small amount of THF added. The mixture was warmed to −20°C within 2 h to complete deprotonation and then cooled to −70°C. Freshly distilled propargylic bromide (0.105 mol) was added in one portion and the solution stirred for 2 h and allowed to come to −50°C. After quenching the reaction with 100 ml of 10% hydrochloric acid (HCl), the layers were separated and the water layer extracted twice with diethyl ether. The combined organic layers were washed with a water and saturated sodium chloride solution, and dried over sodium sulphate. After evaporation of the solvent, the crude product was distilled over a vigreux column under reduced pressure.

2.1.2. Hydrolysis

The 2-(2-propynyl)-alkanoic acid methyl ester (0.03 mol) was dissolved in 60 ml methanol and 20 ml water and stirred with an equimolar amount of lithium hydroxide containing one molecule crystal water for 24 h at room

temperature. The methanol was removed by evaporation, and the residue diluted with water and extracted twice with ether. The water layer was then acidified with concentrated HCl and extracted again with ether. The dried ether solution was freed from solvent and the crude product purified by distillation in vacuo and a chromatographic procedure using silica and a hexane/ethyl acetate (2:1) eluent.

2.1.3. Chromatographic procedures

Thin layer chromatography (TLC) was performed using silica gel plates (Merck, Kieselgel 60 F_{254}) with *n*-hexane/ethyl acetate, in a ratio of 10:1 in the case of the methyl esters and 2:1 for the acids, employed as the mobile phase. After drying, the plates were sprayed with a mixture of 2 ml 4-methoxy-benzaldehyde and 2 ml sulphuric acid in 200 ml methanol and heated for 1 min at 150°C. The methyl esters appeared as blue spots and the carboxylic acids as red-brown loops. Preparative chromatography was performed with silica gel (0.040–0.063 mm, 230–400 mesh; Merck) by flash chromatography and the eluents described for TLC.

2.1.4. Spectral analysis

In proof of chemical structure of the compounds nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AC 250 MHz spectrometer using tetramethylsilane as internal standard. Chemical purity was assessed by gas chromatography–mass spectroscopy (GC/MS) recording the total ion chromatogram and the base-peak that was in all cases the M-15 ion. GC/MS was performed by previously published methods (Fischer et al., 1992). A Hewlett Packard System (type HP 5890 A gas chromatograph; type 5971 MSD mass spectrometer) operated by a MS Chem Station was employed. Compounds were dissolved in acetonitrile and treated with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA; Pierce, Bender and Hobein, München, Germany) for 2 h at room temperature. GC separations were achieved using a 50×0.2 mm i.d. HP-1 capillary column (0.11 μm film thickness) with helium as the carrier gas (0.5 ml/min). The initial temperature of 70°C was held for 1 min, and then raised by 10°C/min to 170°C. The injector temperature was 250°C. The mass spectrometer (electron impact) was operated in scan mode ($m/z = 60$–270). The relative intensities of the various ions were related to the TMS ion ($m/z = 73$). These procedures were used to determine the following characteristics of the analogues synthesised:

Propyl-4-yn-valproic acid (2-Propyl-4-pentynoic acid): bp. (Me-ester): 80°C/12 mbar; bp. (acid): 61–63°C/0.2 mbar; overall yield: 32%. ^{1}H-NMR ($CDCl_3$): $\delta = 0.94$ (^{3}H, t, $J \approx 7.5$ Hz; CH_3), 1.39 (^{2}H, m, CH_2), 1.69 (^{2}H, m, CH_2), 2.02 (^{1}H, t, $J \approx 2.5$ Hz; C≡CH), 2.44 (^{2}H, m, $CH_2C{\equiv}C$), 2.62 (^{1}H, m, CH), 12.0 (^{1}H, broad, COOH).

Butyl-4-yn-valproic acid (2-[2-Propynyl]-hexanoic acid): bp. (Mester): 103–104°C/12 mbar; bp. (acid): 80–82°C/0.2 mbar; overall yield: 24%. ^{1}H-NMR ($CDCl_3$): $\delta = 0.9$ (^{3}H, t, $J \approx 8$ Hz; CH_3), 1.35 (^{4}H, m, $2 \times CH_2$), 1.7 (^{2}H, m, 3-CH_2), 2.02 (^{1}H, t, $J \approx 2$ Hz; C≡CH), 2.42 (^{3}H, m, CH, $CH_2C{\equiv}C$), 11.03 (^{1}H, broad, COOH).

Pentyl-4-yn-valproic acid (2-[2-Propynyl]-heptanoic acid): bp. (Mester): 43–46°C/0.5 mbar; bp. (acid): 82–84°C/0.2 mbar; overall yield: 22%. ^{1}H-NMR ($CDCl_3$): $\delta = 0.92$ (^{3}H, t, $J \approx 7.5$ Hz; CH_3), 1.34 (^{6}H, m, $3 \times CH_2$), 1.68 (^{2}H, m, 3-CH_2), 2.02 (^{1}H, t, $J \approx 2$ Hz; C≡CH), 2.5 (^{3}H, m, CH, $CH_2C{\equiv}C$), 9.1 (^{1}H, broad, COOH).

Hexyl-4-yn-valproic acid (2-[2-Propynyl]-octanoic acid): bp. (Me-ester): 53–55°C/0.5 mbar; bp. (acid): 87°C/0.2 mbar; overall yield: 20%. ^{1}H-NMR ($CDCl_3$): $\delta = 0.9$ (^{3}H, t, $J \approx 8$ Hz; CH_3), 1.32 (^{8}H, m, $4 \times CH_2$), 1.72 (^{2}H, m, 3-CH_2), 2.0 (^{1}H, t, $J \approx 2$ Hz; C≡CH), 2.48 (^{3}H, m, CH, $CH_2C{\equiv}C$), 11.32 (^{1}H, broad, COOH).

Heptyl-4-yn-valproic acid (2-[2-Propynyl]-nonanoic acid): bp. (Me-ester): 59–62°C/0.5 mbar; bp. (acid): 93°C/0.1 mbar; overall yield: 16%. ^{1}H-NMR ($CDCl_3$): $\delta = 0.88$ (^{3}H, t, $J \approx 7.5$ Hz; CH_3), 1.32 (^{10}H, m, $5 \times CH_2$), 1.72 (^{2}H, m, 3-CH_2), 2.02 (^{1}H, t, $J \approx 2$ Hz; C≡CH), 2.5 (^{3}H, m, CH, $CH_2C{\equiv}C$), 10.44 (^{1}H, broad, COOH).

2.2. Determination of analogue activities in vivo and in vitro

2.2.1. Teratogenicity

The induction of exencephaly was determined using NMRI-mice (Harlan-Winkelmann, 33176 Borchen, Germany) as described previously (Nau and Löscher, 1986; Nau, 1986a,b). The animals were kept under controlled conditions: room temperature (21 ± 1°C), relative humidity (50 ± 5%), and a 12-h light–dark cycle with the light period from 1000–2200 h. Females weighing 28–36 gm were allowed to mate with males of the same strain over a 3-h period (0600–0900 h). The animals with vaginal plugs were separated, and the following 24-h period was designated as day 0 of pregnancy. The compounds were injected intraperitoneally on the morning of day 8 of gestation as the sodium salt of each carboxylic acid dissolved in water. All compounds were used as their racemates. On day 18 of gestation the dams were sacrificed, the uteri removed, and the number of implantation sites recorded. Each fetus was weighed and inspected for the presence of external malformations. The animals were given free access to food (Altromin 1324 diet; Lage, Germany) and tap water. Approval for the study was obtained from the Senatsverwaltung für Gesundheit von Berlin, approval number G0410/95.

2.2.2. Estimation of cell proliferation rate

The mouse neuro-2a neuroblastoma and rat C6 glioma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) containing 10% foetal calf serum (Gibco Biocult and Tissue Culture Services, UK), 2 mM glutamine and 100 μg gentamicin/ml (Sigma Chemical, UK) and incubated in 75 cm^2 tissue

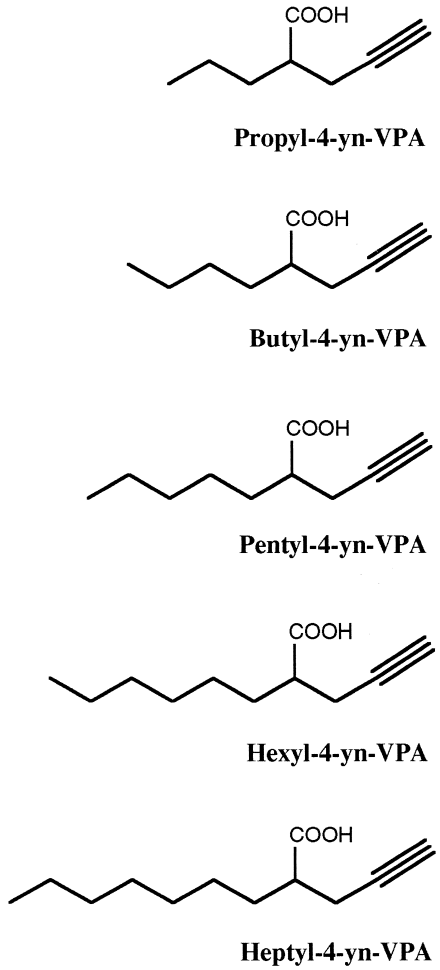

Fig. 1. Structure of valproic acid-related analogues.

culture flasks maintained at 37°C in a humified atmosphere of 9% CO_2. Cells were passaged using 0.025% trypsin in DMEM and were seeded at a density of 1×10^4 cells/cm^2. Antiproliferative effects were assessed in C6 glioma by counting cell number using a nuclei staining procedure (Sanford et al., 1951). Freshly trypsinized cells were seeded (10^4/cm^2) into multiwell dishes. They were allowed to recover for 24 h before being exposed to separate serial concentrations of the analogues for 48-h period. The analogues were dissolved in dimethylsulphoxide (DMSO; Sigma) at a concentration which never exceeded 1% of the tissue culture medium. Following exposure, nuclei counting was performed in duplicate by replacing the medium with 500 μl of crystal violet (0.2%(w/v)) in citric acid (0.1 M). The cells were incubated with this nuclei releasing and staining solution for 30 min at 37°C. Then gentle trituration with a Gilson micropipette was performed in order to obtain an even suspension of stained nuclei. Nuclei were counted using a haemocytometer and the results expressed as a percentage of control values. The concentration of analogue that inhibited the proliferation of C6 glioma by 50% was defined as the IC_{50} and these were determined using a line fitting quadratic equation (Cricket Graph®).

The antiproliferative potential of the valproic acid-related analogues was also evaluated in the L929 fibroblastoid cell line (L-cells) by measuring the rate of 5-bromo-2-deoxyuridine (BrdU) incorporation. The L-cells were purchased from the European Collection of Animal Cell Cultures (Porton Down, UK) and maintained routinely in DMEM with 1 mM sodium pyruvate and 2 mM GlutaMAX™ (Gibco BRL, Denmark), and supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco BRL), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml). Cell lines were maintained in 25 cm^2 flasks (NUNC, Denmark) in 5 ml medium per flask and incubated at 5% CO_2. Cells were passaged using trypsin–EDTA solution (Gibco BRL). The Biotrak™ cell proliferation ELISA system (Amersham Denmark Aps) was used for determination of BrdU incorporation according to manufacturer's procedure. Briefly, L-cells were dislodged with a trypsin–EDTA solution (Gibco BRL) and plated at 5000 to 7000 cells/well in a 96-well culture plate with 10 mM BrdU and test compounds or controls. Cells were grown for 20–24 h and the level of incorporated BrdU was determined by using peroxidase-labeled anti-BrdU antibodies in an ELISA procedure. The effect of each compound was determined at least three times. Means, standard deviations, standard errors, and differences be-

Table 1
Teratogenicity of valproic acid analogues

Compound	Dose (mmol/kg)[a]	No. of dams	Live fetuses	Fetal weight (g)	Embryo lethality (%)[b]	Exencephaly (%)[c]
Propyl-4-yn-valproic acid	1.23	13	89	1.03 ± 0.13	16	12
Butyl-4-yn-valproic acid	1.25	9	63	0.99 ± 0.09	45	71
Pentyl-4-yn-valproic acid	1.25	6	44	1.02 ± 0.09	40	60
Hexyl-4-yn-valproic acid	1.25	7	29	0.96 ± 0.06	67	79
Heptyl-4-yn-valproic acid	1.25	5	5[d]	0.94 ± 0.11	92	80
Controls		14	152	1.19 ± 0.12	11	0

[a] The doses are given in millimole sodium salt of each carboxylic acid per kilogram. All substances were injected intraperitoneally on the morning of day 8 of gestation.
[b] Resorptions and dead fetuses as percentage of total implants.
[c] Percentage of live fetuses.
[d] Four dams which received 1.25 mmol/kg died.

tween data evaluated by the Student's *t*-test were calculated using STATISTICA software.

2.2.3. Qualitative evaluation of neuritogenesis

Neuro-2a cells were grown in 25 cm^2 flasks as previously described. Following a 48-h exposure period to the relevant analogue, the cells were fixed overnight at 4°C in a solution of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.4. The cells were post-fixed in phosphate-buffered 1% osmium tetroxide for 1 h at room temperature, washed and dehydrated gradually for 1 h using a series of ethanol concentrations stepwise through 20, 40, 60, and 80 to a final concentration 100%. Disks with attached cells were cut from of the base of the tissue culture flask and critical point dried to minimize shrinkage and cracking. This was achieved by placing the samples in a Polaron critical point dryer and purging the chamber several times with CO_2 to remove all traces of ethanol. After 1 h the temperature and pressure were increased to 40°C and 1200 lbs/$in.^2$ respectively, at which stage the critical point for carbon dioxide had been reached and drying was completed. Subsequently, the specimens were removed from the chamber, mounted on stubs using conductive carbon cement and sputter coated with gold under vacuum (5×10^{-2} Torr) and in the presence of argon gas at a current of 20 mA for 3 min (Polaron E5100). The samples were examined in the scanning electron microscope (JEOL 35C) at an accelerating voltage of 15 kV.

2.2.4. Quantitative estimation of neural cell adhesion molecule (NCAM) immunofluorescence

Neuro-2a cells were seeded in 24-well plates at a density of 10^4 cells/cm^2 and cultured as described above. Following a 48-h exposure period to the relevant analogue, the cells were fixed progressively by six 10-min incubations with DMEM containing increasing concentrations of neutral buffered formalin stepwise from 10, 30, 50, 70 and 90 to a final concentration of 100%. When fixation was complete, the cells were washed three times over a 30-min period with phosphate buffered saline at pH 7.4. The cells were then incubated with a 1 in 50 dilution of rabbit anti-NCAM antibody (Plioplys et al., 1990) in phosphate buffered saline containing 1% (w/v) bovine serum albumin (Sigma) for 1 h at room temperature and washed three times over a 30-min period with phosphate buffered saline at pH 7.4. The washed cells were then incubated for 1 h at room temperature with a rhodamine-conjugated anti-rabbit antibody (Sigma) diluted 1 in 50 in phosphate buffered saline containing 1% (w/v) bovine serum albumin. The cells were again washed three times with phosphate buffered saline at pH 7.4 before being mounted in Citifluor® (Agar, UK), a fluorescence-enhancing medium. Fluorescence was visualized with a Leitz DMRB fluorescence microscope using an exciting wavelength of 552 nm and an emission wavelength of 570 nm. The relative fluorescence intensity was measured at points of cell–cell contact as grey level units using a Quantimet 500 Image Analysis System. Approximately sixty points of cell contact in randomly chosen areas was determined and the mean estimated. The means from at least three independent experiments were used to calculate the final mean ± S.E.M.

Table 3
Median teratogenic dose (ED_{50}) and slope of teratogenic regression line of valproic acid-analogues[a]

Compound	ED_{50} (mmol/kg)	Slope of regression line
Propyl-4-yn-valproic acid	1.65 (1.57–1.74)[b]	1.35 (1.28–1.44)[b]
Hexyl-4-yn-valproic acid	0.86 (0.67–1.11)	1.38 (1.26–1.51)
Heptyl-4-yn-valproic acid	0.96 (0.85–1.10)	1.27 (1.15–1.41)

[a] Results are calculated according to the work of Litchfield and Wilcoxon (1949).
[b] 95% Confidence interval.

3. Results

The structural features of the analogues synthesised and employed in this study are illustrated in Fig. 1. When compared to control litters, all analogues proved to be teratogenic as they significantly increased the incidence of neural tube defects in the embryos of dams exposed to a single dose of 1.25 mmol/kg on day 8 of gestation (Table 1). Moreover, these effects could not be attributed to impaired growth rates, as there was no significant difference in the mean weight of exposed embryos when com-

Table 2
Dose-dependent teratogenicity of hexyl-4-yn-valproic acid

Compound	Dose (mmol/kg)[a]	No. of dams	Live fetuses	Fetal weight (g)	Embryo lethality (%)[b]	Exencephaly (%)[c]
Hexyl-4-yn-valproic acid	1.5	6	12	0.86 ± 0.07	84	100
	1.25	7	29	0.96 ± 0.06	67	79
	1.0	7	37	1.03 ± 0.05	54	70
	0.75	6	53	1.18 ± 0.11	32	23
	0.5	13	165	1.25 ± 0.10	18	7

[a] The doses are given in millimole sodium salt of each carboxylic acid per kilogram. All substances were injected intraperitoneally on the morning of day 8 of gestation.
[b] Resorptions and dead fetuses as percentage of total implants.
[c] Percentage of live fetuses.

Table 4
Antiproliferative potential of valproic acid analogues in C6 glioma

Compound	Dose					
	0.3 mM	0.6 mM	1.2 mM	1.8 mM	2.4 mM	3.0 mM
Propyl-4-yn-valproic acid	89 ± 2	79 ± 3	56 ± 4	41 ± 4	18 ± 3	6 ± 2
Butyl-4-yn-valproic acid	100 ± 10	94 ± 15	45 ± 10	18 ± 2	9 ± 3	8 ± 3
Pentyl-4-yn-valproic acid	77 ± 5	52 ± 7	24 ± 4	4 ± 1	1 ± 1	0
Hexyl-4-yn-valproic acid	49 ± 4	16 ± 4	5 ± 1	2 ± 6	1 ± 1	0
Heptyl-4-yn-valproic acid	16 ± 3	10 ± 6	1 ± 0	1 ± 0	0	0

Cell number was estimated by direct counting. Values are expressed as percent control and are the mean ± S.E.M. ($n = 3$).

pared to their control counterparts, and occurred in a dose-dependent manner, as evidenced by the correlation observed between increasing doses of hexyl-4-yn-valproic acid and rate of exencephaly (Tables 1 and 2). Litchfield–Wilcoxon analysis of dose–response effects between propyl-4-yn-valproic acid and hexyl-4-yn-valproic acid revealed the curves to be parallel and allowed a potency ratio of 1.91 to be calculated (Table 3). In contrast to this two-fold increase in potency between propyl-4-yn-valproic acid and hexyl-4-yn-valproic acid, no difference was noted between hexyl-4-yn-valproic acid and heptyl-4-yn-valproic acid (potency ratio: 1.05), thereby demonstrating the teratogenicity of these analogues to increase with increasing length of the *n*-alkyl side chain up to hexyl-4-yn-valproic acid. A corresponding increase in embryolethality was also observed and, with heptyl-4-yn-valproic acid, this toxicity was observed in the dams.

As increasing chain length enhanced the teratogenic potential of these analogues, their ability to inhibit proliferation rate in C6 glioma was investigated. Dose-dependent effects were evaluated over a concentration range of 0–3 mM following a 48-h exposure, which is twice the normal doubling time for this cell line. Moreover, the concentration range employed encompasses the serum therapeutic range of the valproate parent compound and that expected to accumulate in the embryo (Wilder and Karas, 1982; Nau, 1986a). In all cases, these analogues significantly inhibited cell proliferation rate within the concentration range evaluated (Table 4). By using quadratic curve fitting to estimate IC_{50} values, their antiproliferative potential could be ranked as heptyl-4-yn-valproic acid (0.14 mM) > hexyl-4-yn-valproic acid (0.82 mM) > pentyl-4-yn-valproic acid (0.98 mM) > butyl-4-yn-valproic acid (1.15 mM) > propyl-4-yn-valproic acid (1.32 mM). Thus, extending ana-

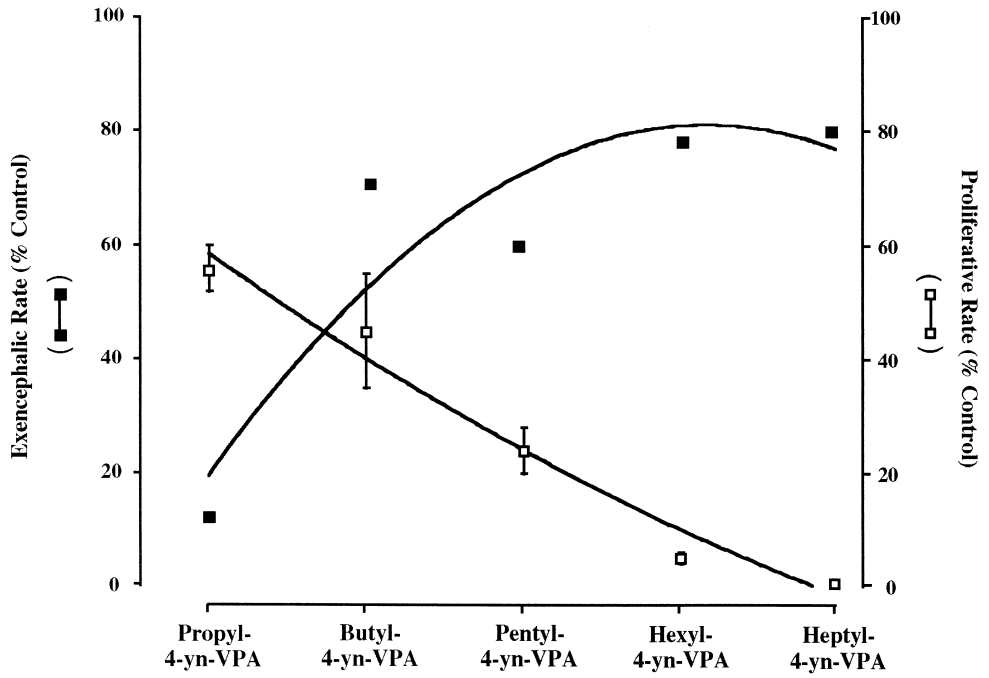

Fig. 2. Influence of analogue *n*-alkyl chain length on induction of exencephaly and inhibition of proliferative rate. Pregnant mice were exposed separately to a single dose (1.25 mmol/kg) of each analogue on day 8 of gestation and the incidence of exencephaly determined on gestation day 18. The antiproliferative rate was determined in C6 glioma following a 48-h exposure to 1.2 mM of each analogue. The data employed were derived from Tables 1 and 2.

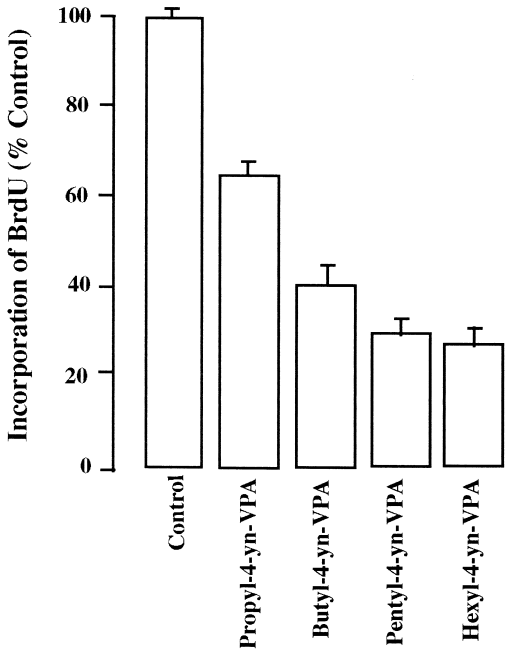

Fig. 3. Inhibition of BrdU incorporation by valproic acid analogues in L929 cells. Values are expressed as the mean ± S.E.M. ($n = 3$).

logue chain length results in a progressive depression of proliferative rate in vitro and a corresponding rise in in vivo teratogenicity. This inverse relationship is illustrated in Fig. 2 in which antiproliferative potential is compared to exencephalic rate at single dose for each analogue, 1.2 mM and 1.25 mmol/kg, respectively. The teratogenicity observed with analogues containing *n*-alkyl side chains in excess of 5 carbon atoms most likely reflects their concomitant increase the overall in vivo toxicity. In contrast, no toxic effects were noted in vitro and proliferative potential continued to be progressively impaired with increasing chain length.

The antiproliferative effects observed with these analogues was not cell type specific as similar results were obtained by determining the rate of BrdU incorporation into L-cells. Following separate 24-h exposures to the same concentration (3 mM) of each analogue, the same ranking of antiproliferative potential was established (Fig. 3). Based on IC_{50} values, these could be ranked as hexyl-4-yn-valproic acid (0.7 ± 0.12 mM) > pentyl-4-yn-valproic acid (2.1 ± 0.15 mM) > butyl-4-yn-valproic acid (2.7 ± 0.2 mM) > propyl-4-yn-valproic acid (7.3 ± 0.8 mM). These effects are equivalent to those obtained by the nuclei counting procedure albeit not as profound given that the exposure protocol was twice that employed in the BrdU experiments. Moreover, the continuing ability of these cells to incorporate BrdU further confirms the lack of any cytotoxicity.

The neuro-2A neuroblastoma was employed to determine if the antiproliferative action of these analogues was associated with morphological change, as has been demonstrated previously following exposure to valproic acid (Regan, 1985). Control cells exhibited a characteristic rounded morphology with numerous villi projecting from the cell surface. Following a 48-h exposure, all analogues induced profound shape changes in the neuro-2A neuroblastoma within the concentration range employed to determine their antiproliferative effects. The cells became flattened, suggesting a greater adherence to the substratum, and produced numerous neurites which formed extensive networks, as evidenced following exposure to 1 mM pentyl-4-yn-valproic acid (Fig. 4). Many of these processes had associated areas of cytoplasmic extensions not unlike those often associated with the filopodial extensions of

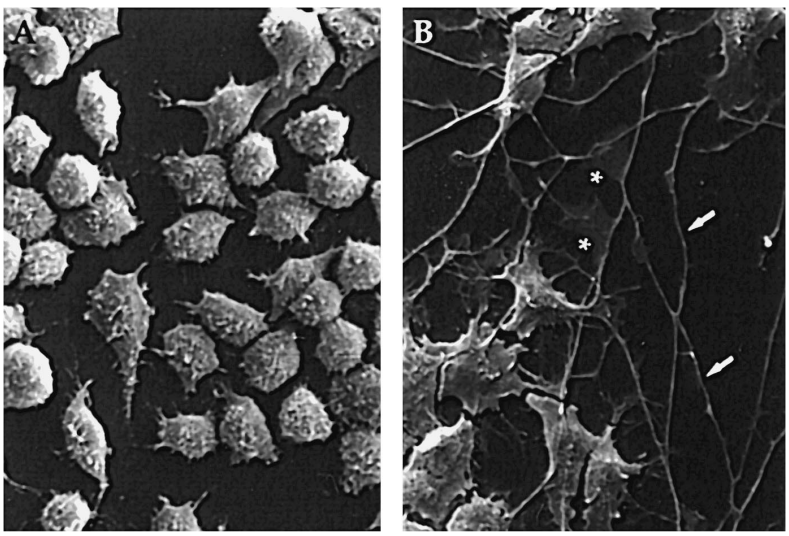

Fig. 4. Analogue-induced neuritogenesis in neuro-2A neuroblastoma. The scanning electron micrographs represent control cells exposed for 48 h to vehicle alone (Panel A) and 1 mM pentyl-4-yn-valproic acid (Panel B). The arrows and asterisks indicate the extent of neuritic arbor and associated cytoplasmic extensions, respectively.

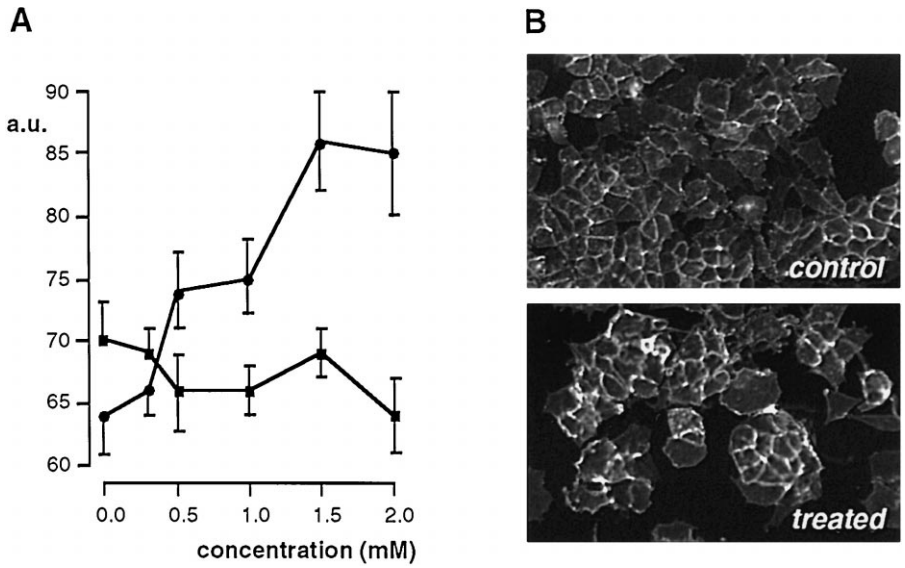

Fig. 5. Analogue-induction of NCAM prevalence in neuro-2a neuroblastoma. The dose-dependent effect of butyl-4-yn-valproic acid and pentyl-4-yn-valproic acid on NCAM prevalence is indicated in Panel A. Values are expressed as the mean ± S.E.M. ($n = 3$) of grey level arbitrary units (a.u.) of relative fluorescence intensity at points of cell–cell contact in control cells and those exposed separately to increasing concentrations of butyl-4-yn-valproic acid (squares) and pentyl-4-yn-valproic acid (circles) for a 48-h period. Panel B shows NCAM immunoreactivity pattern in control cells and those exposed to 1 mM pentyl-4-yn-valproic acid.

growth cones in vitro. To determine if this neuritogenic response was associated with altered NCAM prevalence, immunohistochemistry was employed using an antibody specific for all three major isoforms of this CAM (Pliophys et al., 1990). NCAM immunoreactivity could be detected only at points of cell–cell contact, as has been described previously (Bloch, 1992), and was not apparent on the associated processes (Fig. 5). At points of contact an apparent increase in NCAM immunoreactivity was observed in cells exposed to 1 mM pentyl-4-yn-valproic acid for a 48 h period. Direct quantification of fluorescence intensity at points of cell–cell contact confirmed that this analogue increased NCAM prevalence in a dose-dependent manner over a 0–2 mM concentration range (Fig. 5). This was not observed with butyl-4-yn-valproic acid suggesting that the extension of the *n*-alkyl chain to five carbon units results in a significant alteration in the biological response to this analogue.

4. Discussion

Introduction of a triple bond into one of the chains of valproic acid has produced the most potent teratogenic analogue observed to date, an advance that has proved crucial to understanding key elements of the essential pharmacophore (Hauck and Nau, 1989; Nau et al., 1991). Importantly, this substitution created a chiral centre on carbon atom C2 and demonstrated the importance of a stereospecific site within the embryo at which only the *S*-enantiomer is effective. The biological consequences of interactions at these sites remain to be established but must impinge on molecular cascades that mediate the antiproliferative and prodifferentiative effects observed in vitro. These are critical to the orderly assembly of the developing embryo and are differentially affected in a stereospecific manner. For example, an antiproliferative action combined with a prodifferentiative effect, the latter measured by change in lectin affinity (Braun et al., 1982), depends on the presence of a carboxylic acid group, alkyl chains containing three carbon atoms and an α-hydrogen in a sp^3 configuration (Maguire and Regan, 1991; Regan et al., 1991; Courage-Maguire et al., 1997), features proposed as essential for in vivo teratogenicity (Nau et al., 1991). Moreover, analogue-induced change in cell shape, motility and neuritogenesis is specific to the teratogenic *S*-enantiomer alone (Berezin et al., 1996; Bacon et al., 1998).

These combined observations have contributed to a hypothetical model on the nature of a stereospecific site within the embryo through which these analogues mediate their teratogenic action (Fig. 6). In this model, the *n*-alkyl side is proposed to serve as an ‘anchor’ within a hydrophobic pocket in order to facilitate the ionic and/or H-bonding of the carboxylic acid and, in the case of the 4-yn-valproic acid analogues, the high electron density of the carbon–carbon triple bond. This hydrophobic pocket appears to be particularly important as methylation of the appropriate alkyl chain eliminates teratogenicity (Bojic et al., 1996). The present results contribute further to a basis for this putative site as, with increasing chain length, teratogenicity dramatically increases presumably due to greater binding of the analogue within it. This is also reflected in vitro, as depression of proliferative rate is directly correlated with increasing *n*-alkyl chain length of

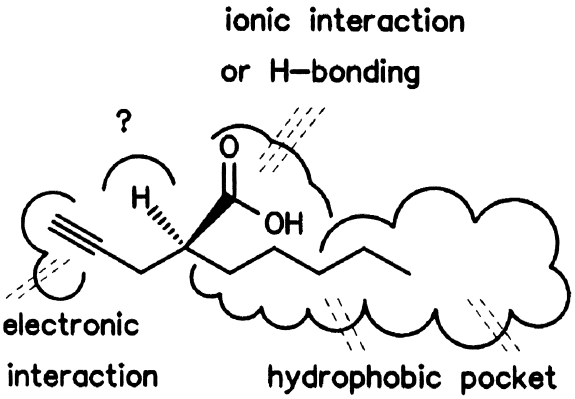

Fig. 6. Hypothetical binding site for valproic acid and related analogues.

the analogues. This latter structure–activity relationship may be the sole determinant of antiproliferative potential as previous studies have indicated unbranched carboxylic acids, such as octanoic acid, to exert most profound inhibitory effects on cell division (Courage-Maguire et al., 1997). Given that octanoic acid is rapidly eliminated in vivo and fails to produce a teratogenic response (Scott et al., 1994), the importance of the *n*-alkyl side chain in effecting binding is further confirmed, as is the importance of the C2 atom and its substituents in mediating teratogenicity.

The coupled antiproliferative and prodifferentiative actions of valproic acid and related analogues are dependent on structure–activity principles similar to those required for in vivo teratogenicity (Regan et al., 1991; Courage-Maguire et al., 1997). The cell shape changes associated with these prodifferentiative actions have been observed to occur in a stereospecific manner in a variety of cell types and to be independent of cell substrate factors (Regan, 1985; Martin et al., 1988; Berezin et al., 1996; Bacon et al., 1998). Moreover, the dose-dependent induction of NCAM prevalence appears to be associated with more potent teratogens, such as the pentyl-4-yn-valproic acid analogue, as similar effects have been obtained previously with valproic acid and retinoic acid, teratogens that mediate G1-dependent growth arrest (Haddox and Russell, 1979; Martin and Regan, 1991; Husmann et al., 1989; Cinatl et al., 1996). However, analogue-induced neuritogenesis in the neuro-2A cell line may not necessarily be associated with increased NCAM prevalence as NCAM-dependent neuritogenesis is a feature of primary neurons cultured on living cells expressing NCAM and those cultured on purified NCAM fail to show outgrowth (Bixby et al., 1987; Doherty et al., 1990; Hankin and Lagenauer, 1994). Moreover, such analogues have no effect on integrin-dependent neuritogenesis (Bacon et al., 1998). It is more likely that pentyl-4-yn-valproic acid induced NCAM prevalence facilitates signal transduction events associated with loss of proliferative potential as addition of purified NCAM to neural cells potently inhibits their proliferation, the maximal effect occurring at low cell density (Sporns et al., 1995). The clustering of NCAM at points of cell–cell contact may be a cardinal feature as this is dependent on the extracellular domains which mediate signal transduction events (Bloch, 1992; Sporns et al., 1995; Sandig et al., 1996; Walsh and Doherty, 1997). The signal cascade influenced by valproic acid and related analogues appears to involve indirect inhibition of protein kinase C (PKC), as their inhibition of cell proliferation occurs prior to the mid G1 phase restriction point, the only time at which enzyme inhibition is observed (Martin and Regan, 1988; Chen et al., 1994, 1996; Bacon et al., 1997, 1998; O'Brien and Regan, 1998). Moreover, neuritogenesis occurs in the presence of PKC inhibitors and in cells depleted of PKC function (Rheinhold and Neet, 1989; Felipo et al., 1990).

As classical binding sites are unknown for valproic acid and related analogues (Löscher, 1993) they may operate in a manner not unlike that proposed to underlie the molecular actions of volatile anaesthetics. Indeed, valproate and anaesthetics are analogous in that they exhibit stereoisomerism, an antiproliferative action in the G1 phase of the cell cycle, and indirectly inhibit the regulatory domain of PKC (Sturrock and Nunn, 1976; Franks and Lieb, 1991; Martin and Regan, 1991; Slater et al., 1993; Chen et al., 1994). Given that their actions cannot be attributed to bilayer disruption (Perlman and Goldstein, 1984; Franks and Lieb, 1994), it supports the general concept that hydrophilic molecules can mediate stereospecific effects at defined protein 'receptor' sites.

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