Differentiation of cultured neuroblastoma cells by urea derivatives

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DIFFERENTIATION OF CULTURED NEUROBLASTOMA CELLS BY UREA DERIVATIVES

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1. Introduction

Many neoplastic cell lines can be induced to undergo differentiation in vitro, exhibiting a morphology and biochemical characteristics similar to that of their normal counterparts. In the case of mouse neuroblastoma this is observed as an outgrowth of processes from the cell body and the expression of neural enzymes [1]. Differentiation of neoplastic cells can be induced by a variety of agents, among them DMSO [2] and other cryoprotectants [3,4]. Some of these compounds are urea analogs. During toxicity tests of the herbicide Diuron (fig.1), it was noted that this substance could differentiate the mouse neuroblastoma clone 41A3 [5]. Since Diuron is an aromatic urea derivative, 7 other urea herbicides (Buturon,

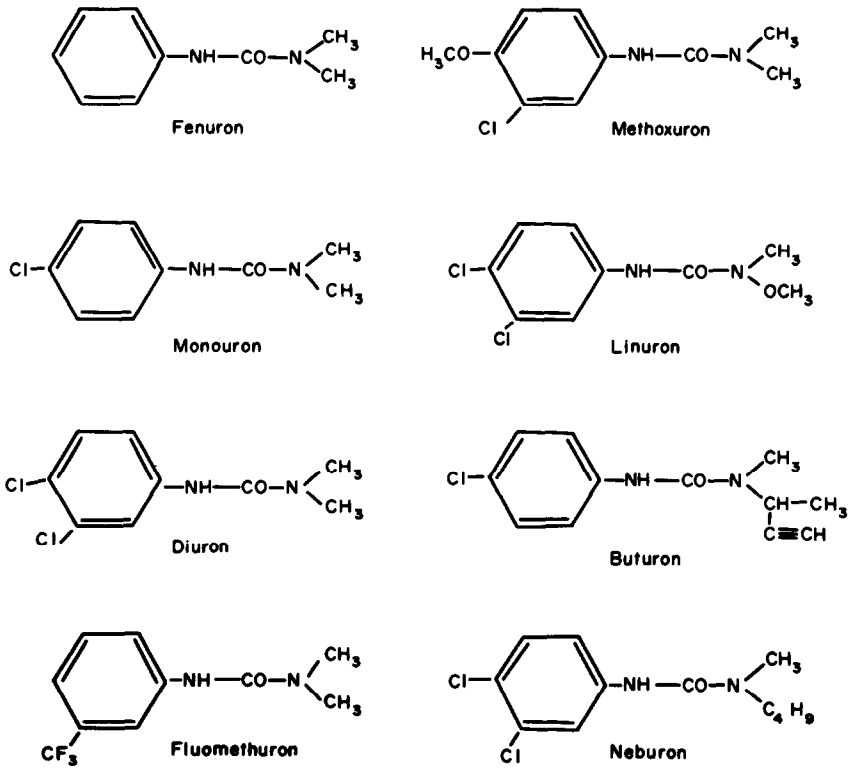

Fig.1. Structural formulae of the urea herbicides tested.

Fenuron, Fluomethuron, Linuron, Methoxuron, Monouron and Neburon; fig.1) and four non-aromatic urea analogs (dimethylurea, ethylurea, tetramethylurea and thiourea) were tested for a possible differentiating effect on mouse neuroblastoma cells.

All aromatic urea derivatives except Fenuron induced morphological differentiation. There was a correlation to the 0.1% level between the differentiating ability of the substances and their octanol/water partition coefficients. The effects of Neburon, Diuron, Monouron and Fenuron on the activity of ecto-acetylcholinesterase, a marker of biochemical differentiation, were investigated. There was an increase in enzyme activity with increasing lipid solubility of the drug used, with a correlation to the 0.5% level. This is interpreted as an indication of a membrane-mediated mode of action.

2. Materials and methods

2.1. *Chemicals*

Ham's F10 medium and foetal bovine serum were obtained from Flow Labs and newborn calf serum from Wallenberg Labs. Penicillin and streptomycin were purchased from Glaxo, the herbicides from Serva Feinbiochemica, acetylthiocholineiodide, dimethylurea and tetramethylurea from Sigma, ethylurea from Riedel-De Haen, and thiourea from Merck.

2.2. *Cell cultures*

The 41A3 clone of the C1300 mouse neuroblastoma was used. The cells were grown as monolayer cultures in glass bottles with Ham's F 10 medium, supplemented with 10% newborn calf serum plus 5% foetal calf serum. The medium contained 100 units penicillin and 50 μg streptomycin/ml. The cultures were kept in an incubator at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed twice a week, and subcultures were made with 0.15% trypsin in a phosphate-buffered salt solution.

2.3. *Experimental procedures*

The cells were trypsinized and plated on Falcon 60 mm plastic tissue culture dishes at 2×10^4 cells/cm. After 2 h, the medium was changed and the test substances were added. Dimethylurea, ethylurea, tetramethylurea and thiourea were dissolved directly in the medium, while the aromatic compounds were added from stock solutions in ethanol. The ethanol concentration in the medium was always $< 0.5\%$. Controls with the herbicides dissolved directly in the medium gave identical results, demonstrating that this amount of ethanol had no effect in itself. Each substance was tested in a 3 μM, 10 μM, 30 μM, . ., series of concentrations. The medium was changed after 24 h and after 48 h, the dishes were scored for differentiation under an inverted microscope. Cells that had developed a process exceeding the diameter of the cell body in length were regarded as differentiated. Cultures with an approximate doubling in the number of differentiated cells as compared to the controls were regarded as differentiated. A detectable increase in the number of detached cells and/or the number of cells stained by Trypan blue was regarded as a sign of toxicity.

Acetylcholinesterase (EC 3.1.1.7.) activity was determined by the method in [6] as used for the measurement of ecto-acetylcholinesterase in intact cells [7]. Protein was determined by the Lowry method [8] using bovine serum albumin as standard.

Partition coefficients were determined by measuring the concentration of the drug in Hank's balanced salt solution before and after extraction with octanol. The concentrations were assayed spectrophotometrically at 245 nm (225 nm for tetramethylurea). The results are given as log P where:

$$P = \frac{C\,(\text{octanol})}{C\,(\text{water})}$$

3. Results

3.1. *Morphological differentiation*

Under normal culture conditions, 41A3 cells differentiate spontaneously to some extent. In contrast to some other neuroblastoma clones, these cells were not differentiated by DMSO. Incubation for 2 days with the different herbicides increased the degree of differentiation, while dimethylurea, ethylurea, tetramethylurea and thiourea had no other effect than an inhibition of the spontaneous differentiation at 30 mM. The concentrations at which a significantly increased differentiation was observed are listed in table 1.

Table 1
Induction of neurite formation in neuroblastoma cells by urea derivatives

Substance	Log P	Conc. μM
Neburon	3.8	30
Linuron	3.2	30
Buturon	3.0	30
Diuron	2.8	30
Fluomethuron	2.2	100
Monouron	1.8	100
Methoxuron	1.5	100
Fenuron	0.9	–
Tetramethylurea	−0.6	–
Thiourea	−1.14[a]	–
Ethylurea	−1.97[a]	–
Dimethylurea	−2.10[a]	–

[a] Data from [15]

The concentrations necessary to give a significant increase in the degree of differentiation of neuroblastoma cells are given in μM. Data from 4 separate experiments. Log P denotes the logarithm of the partition coefficient in an octanol/water system.

Statistical analysis of the correlation between differentiation and log P was performed by means of exact probability for $R \times C$ contingency tables according to [9], giving a value of $p \ll 0.001$.

3.2. *Acetylcholinesterase activity*

Acetylcholinesterase (AchE) is considered to be a biochemical marker of differentiation in mouse neuroblastoma cells [1]. To test for a possible induction of ecto-AchE by the urea herbicides, 4 drugs were chosen in a series with the log P values ~1 unit apart. The AchE activity was determined after incubation with the drug at 100 μM and 300 μM, with the exception of Neburon, which was not soluble at 300 μM. The results are shown in table 2. Analysis of the correlation between log P and enzyme activity gave a value of $p < 0.005$ for the 100 μM series, and $p < 0.002$ for the 300 μM series, using Student's t-test.

Table 2
Induction of ecto-acetylcholinesterase in neuroblastoma cells by urea derivatives

Substance	Conc. (μM)	AchE activity ± SEM (nmol hydrolyzed substrate/mg prot)
Control	–	58.1 ± 2.5
Neburon	100	89.7 ± 7.7
Diuron	100	71.4 ± 6.0
	300	81.1 ± 6.0
Monouron	100	63.5 ± 3.8
	300	68.1 ± 3.3
Fenuron	100	65.6 ± 3.7
	300	50.0 ± 4.8

Each value represents the mean of ⩾ 7 determinations from two separate experiments. For statistical analysis of results, see section 3.2

3.3. *Toxicity*

In accordance with reports on Diuron [5], all the tested herbicides exhibited a cell-density dependent toxicity. Drug concentrations that did not have any toxic or differentiating effect within 48 h at the cell densities used for the differentiation experiments (2×10^4 cells/cm^2) caused cell death of dense cultures ($> 10^5$ cells/cm^2). These and reported results [5] imply that the toxic effects are due to a metabolite rather than to the substance itself, or that the substances interfere with a mechanism that is expressed only in dense cultures.

4. Discussion

Statistical analysis of the results from the morphological studies and the AchE assays (see sections 3.1, 3.2.) indicate a correlation between the lipid solubility and the differentiating ability of a substance. The fact that the more lipid-soluble compounds are the more potent ones indicates that the membranes of the cell might be the sites of action, since there is evidence that the composition of the cell membrane affects the differentiation of the cell. It has been reported that differentiation can be induced in mouse neuroblastoma cells by incubation with phosphatidylcholine–phosphatidylserine liposomes [10] or by culture in delipidated medium [11]. Furthermore, supplementation with oleic acid can block differentiation [11] and general anesthetics [12] as well as local anesthetics [13] can reverse differentiation. It is generally assumed that anesthetics and unsaturated fatty acids increase the fluidity of membranes. If the inhibition of differentiation is caused

by an increase in membrane fluidity, it is tempting to speculate that the differentiating effect of the urea herbicides may be due to a decrease in the fluidity of the membrane, especially since it has been reported that the membrane fluidity of neuroblastoma cells is decreased upon differentiation [14]. Such an assumption finds support in the work carried out on the non-aromatic ureas, known to differentiate Friend leucemic cells. Working with lipid vesicles, calorimetric evidence was reported [4] for a stabilisation of the lipid bilayer, indicating a decrease in membrane fluidity, possibly due to charge interactions and hydrogen bonding. It was pointed out [3] that these non-aromatic ureas can provide electrons for hydrogen bonding. It seems reasonable to assume that the same properties might be shared by the urea herbicides.

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