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CONJUGATION PATHWAYS IN HUMAN BRONCHIAL CARCINOMA CELL LINES

ELIZABETH M. GIBBY,* REKHA MEHTA,† MORAG ELLISON‡ and GERALD M. COHEN*

*Toxicology Unit, Department of Pharmacology, School of Pharmacy, University of London, 29 Brunswick Square, London WC1N 1AX; †Department of Biochemistry, University of Surrey, Guildford GU2 5XH, Surrey; and ‡Ludwig Institute for Cancer Research (London Branch), Royal Marsden Hospital, Sutton, Surrey SM2 5PX, U.K.

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Abstract—Human bronchial carcinoma cell lines in monolayer cell culture were used to study the conjugation of 1-naphthol and 3-hydroxybenzo(*a*)pyrene, two phenolic substrates. Cell lines, able to metabolise 1-naphthol to glucuronic acid and sulphate ester conjugates, showed a predominance of glucuronidation. This is in sharp contrast to normal human peripheral lung which metabolises these phenolic substrates predominantly to the sulphate ester conjugates. The bronchial tumour cell lines do not all have the same conjugation pattern and they differ in the metabolism of phenolic substrates when compared to normal peripheral lung. The cell lines generally show that sulphate ester conjugation was either reduced or absent. In some cases, dependent on the type of tumour the cell line was derived from, glucuronic acid conjugation was elevated. Initial sub-cellular studies with a poorly differentiated epidermoid cell line (Ben) demonstrated that this line had an active UDP-glucuronosyltransferase (K_m 33 μM and V_{max} 1.8 nmoles $min^{-1} \cdot mg\ protein^{-1}$), using 1-naphthol as substrate. However, this cell line was unable to form significant amounts of sulphate ester conjugates, due to either low sulphotransferase activity, or an inability to generate adenosine 3′-phosphate 5′-sulphatophosphate (PAPS).

Lung cancer has produced many fatalities in Western Society [1]. By identifying major differences in metabolic pathways between cancerous and normal tissues, it may be possible to design drugs which are selectively toxic to the tumour tissue. Recent studies, using short-term organ cultures of human peripheral lung and tumour tissue from the same patients, have shown striking qualitative differences in the routes of conjugation of 1-naphthol, a model phenolic substrate. Whereas normal lung tissue formed primarily 1-naphthyl sulphate [2, 3] tumour tissue formed almost exclusively the glucuronic acid conjugate, 1-naphthyl-β-D-glucuronide [3]. Thus, there appears to be a major biochemical difference between normal and cancerous human lung tissue which may be exploitable in chemotherapy. All the tumours which show this phenomenon have been classified histopathologically as poorly differentiated squamous. However, as this is not the only type of carcinoma found in the lung it is important to determine whether similar alterations in the activity of conjugating enzymes are characteristic of other lung tumours. As the availability of surgical specimens of other lung tumour types is limited, cell lines derived from different histological types of lung carcinomas have been used. The ability of these monolayer cell cultures to metabolise 1-naphthol and 3-hydroxybenzo(*a*)pyrene to their respective sulphate and glucuronic acid conjugates has been examined.

Having found a predominance of glucuronidation of 1-naphthol in short-term organ cultures of human tumour tissue [3], it is interesting that in animal tumours, transplantable rat hepatomas have higher UDP-glucuronosyltransferase activity compared to normal liver [4]. This higher UDP-glucuronosyltransferase activity, in Reuber H-35 hepatoma has been noted with several substrates, e.g. *p*-nitrophenol, *o*- and *p*-aminophenol and 1-naphthol [4–7]. Such increases in the conjugating ability of tumours are in contrast to the general observation that the microsomal mixed function oxidation reactions are generally decreased in tumours [8–10].

In the present study, marked variability in the pattern of conjugation was observed in the different cell lines. However, the majority (4/5) of the human lung tumour cell lines, where significant metabolism of 1-naphthol was observed, formed predominantly glucuronic acid conjugates with smaller, or barely detectable amounts of sulphate ester conjugates.

MATERIALS AND METHODS

Cell culture

Eight cell lines, originally derived from human bronchial carcinomas, were used (Table 1). Cells were routinely cultured [11] in 90% Dulbecco's modified Eagle's medium, 10% bovine foetal calf serum, kanamycin (100 mg/l) and fungizone (1.25 mg/l) in plastic tissue culture flasks (Nunc), maintained at 37° in an atmosphere of 10% carbon dioxide in air.

1-Naphthol and 3-hydroxybenzo(a)pyrene conjugation in cell cultures

After having decanted Dulbecco's medium (5 ml) from each flask, 3 ml of the same medium containing either 2 μM[^{3}H]-3-hydroxybenzo(*a*)pyrene (sp. act. 146 mCi/mmole; Midwest Research Institute, Kansas City, MO), or 10 μM [1-^{14}C]-1-naphthol (sp. act. 19.2 Ci/mole; Radiochemical Centre, Amersham, U.K.), was added to the monolayer cell culture. Duplicate incubations of each cell line, with each

Table 1. Characteristics of cell lines used

Cell line	Histological type	Approx doubling time	Passages*
Ben	Poorly differentiated epidermoid	2–3 days	20–40
Gri	Squamous	2 days	30–40
Min	Adenocarcinoma	4 days	10–20
Poc	Oat cell	6–8 weeks	2nd and third
Mar	Oat cell	6–7 days	18–22
Mor	Large cell anaplastic	2–3 days	25–35
Dre	Large cell anaplastic	2 days	25–35
E14	Squamous	3 days	>70

* No. of passages through which the cells were taken during these experiments.

substrate, were carried out at 37° for 90 min. At the end of this time period the medium was decanted from the cells and stored frozen until analysed.

The cells ($0.1–0.5 \times 10^7$) from each incubation were lysed by the addition of 3 ml of 0.1 M sodium hydroxide. Protein was determined, on these lysates of the cells using bovine serum albumin as a standard, using Lowry's method [13].

1-Naphthol conjugation by subcellular fractions

A 50% homogenate of either Ben cells or E14 cells was made in 1.15% potassium chloride. The homogenates were centrifuged in a Mistral High Speed 18 at 10,000 *g* for 30 min and the resulting supernatant fractions used to assess either sulphate ester or glucuronic acid conjugation. In the latter case, the reaction mixture (total volume 500 μl) consisted of either 0.15 mg protein from the Ben cells or 0.07 mg protein from E14 cells; [1-^{14}C]-1-naphthol at a range of concentrations from 2–20 μM; 1.5 mM UDP-glucuronic acid in 0.05 M Tris–HCl pH 7.4 containing 4 mM magnesium chloride (final concentrations). To measure sulphation, the reaction mixture (total volume, 500 μl) consisted of either 0.15 mg protein from the Ben cells or 0.07 mg protein from E14 cells; [1-^{14}C]-1-naphthol at a range of concentrations from 2–20 μM; 2 mM sodium sulphate; 5 mM magnesium chloride; 5 mM ATP and 0.05 M Tris–HCl pH 7.4 (final concentrations). The reactions were initiated by the addition of enzyme and incubated at 37° in a shaking water bath for 10, 30 or 60 min with Ben cells and 60 min with E14 cells.

In order to terminate the reaction, 100 μl of the incubation medium was removed and added to 100 μl of methanol containing the unlabelled standards: 1-naphthol (Hopkin and Williams Ltd., Chadwell Heath, U.K.), 1-naphthyl-β-D-glucuronide (Koch-Light Laboratories Ltd., Colnbrook, U.K.), and 1-naphthyl sulphate (potassium salt from Sigma Chemical Company, London, U.K.).

Chromatography of 1-naphthol conjugates

The conjugates in the media were analysed by thin layer chromatography essentially as described by Mehta *et al.* [12]. Aliquots (50 μl) of the culture medium from one incubation together with 50 μl of authentic standards of 1-naphthyl-β-D-glucuronide, 1-naphthyl sulphate and 1-naphthol were applied to silica gel t.l.c. plates (200 × 400 mm; Kieselgel 60F_{254}, 0.2 mm thickness, aluminium-backed plates from E. Merck, Darmstadt, W. Germany) and the metabolites separated using a solvent system of toluene:glacial acetic acid:acetone (2:1:2 by volume). The metabolites associated with radioactivity were detected by radiochromatogram scanning (Varian-Berthold Radiochromatogram Scanner). The quantitation of the conjugates present in each medium was carried out by cutting the chromatogram at the position of the fluorescent standards, followed by liquid scintillation counting in a scintillant containing 0.5% w/v PPO, 0.02% w/v dimethyl POPOP, 33% v/v Metapol (Durham Chemicals Distributors Limited, Birtley, Tyne and Wear, U.K.) in toluene, using an LKB Rackbeta 1216 scintillation counter. The amounts of radioactivity (<1% of total radioactive label running with fluorescent standards) detected at similar *Rf*'s as the conjugates in the appropriate controls were subtracted from the corresponding values obtained in the experimental determinations.

The analysis of the 1-naphthol conjugates formed by the subcellular fractions was carried out as above but without the radiochromatogram scanning.

Chromatography of 3-hydroxybenzo(a)pyrene (3-OH-BP) conjugates

The conjugates in the culture media were analysed by t.l.c. An equivolume mixture of culture medium and methanol:acetone (4:1 by volume) was made up together with unlabelled standards of 3-OH-BP and 3-OH-BP-β-D-glucopyranosiduronic acid, benzo(*a*)pyren-3-yl-hydrogen sulphate kindly provided by the Chemical Repository, IIT Research Institute, Chicago, IL). Aliquots (100 μl) were applied to non-fluorescent silica gel t.l.c. plates (200 × 40 mm; Kieselgel 60, 0.2 mm thickness, aluminium-backed plates without fluorescent indicator from E. Merck, Darmstadt, W. Germany) and the metabolites were separated using a solvent system of propan-1-ol:ammonia:toluene (7:3:1 by volume). The quantitation of the conjugates was carried out in the the same manner as that described for 1-naphthol. The amounts of radioactivity (<3% of total radioactive label running with fluorescent standards) detected at similar *Rf*'s as the conjugates in the appropriate controls were subtracted from the

corresponding values obtained in the experimental determinations.

RESULTS

Metabolism of [1-^{14}C]-1-*naphthol by cell lines*

The routes and rates of formation of the conjugates of 1-naphthol by eight different cell lines were very variable (Table 2). Two cell lines, Ben and Gri, derived from squamous tumours formed predominantly the glucuronic acid conjugate whilst another squamous cell line E14 showed virtually no metabolism. Min, the adenocarcinoma and Mor, one of the large cell anaplastic cell lines both showed significantly more glucuronic acid than sulphate ester formation. The other large cell anaplastic cell line, Dre, formed detectable amounts of 1-naphthyl sulphate only. Both the two oat cell carcinoma cell lines, Poc and Mar, showed virtually no metabolism of 1-naphthol.

Metabolism of [^{3}H]-3-*hydroxybenzo(a)pyrene (3-OH-BP) by cell lines*

For all the cell lines studied, the metabolism of 3-OH-BP was lower than that observed for 1-naphthol (Table 2). Conjugation of 3-OH-BP was fastest with the cell line Ben, in agreement with the results with 1-naphthol. With this cell line conjugation with glucuronic acid was more extensive than with sulphate. Gri forms small quantities of both conjugates. Min also produced both conjugates, but only trace amounts of 3-OH-BP-β-D-glucuronide. Small, but detectable amounts of the glucuronide conjugate were formed by Mar, the oat cell carcinoma cell line. The Dre cell line produced trace amounts of 3-OH-BP-β-D-glucuronide and a small quantity of 3-OH-BP hydrogen sulphate, while the other large cell anaplastic line metabolised the 3-OH-BP to the sulphate ester conjugate only. E14 showed virtually no metabolism, although traces of sulphate were detected.

Metabolism of 1-naphthol by subcellular fractions from the cell lines Ben and E14

In order to confirm and extend the above results, the conjugation of 1-naphthol by Ben and E14 was also studied using subcellular fractions prepared as described in Materials and Methods. Using 10,000 *g* supernatant fraction from the Ben cell line together with appropriate co-factors, 1-naphthol was metabolised to 1-naphthyl-β-D-glucuronide in a time, substrate and enzyme-dependent manner. A Lineweaver–Burk plot was drawn from the subcellular kinetic studies carried out with the Ben cells for glucuronidation of 1-naphthol. From this a value for V_{max} of 1.8 nmoles mg protein$^{-1}\cdot$min^{-1} and an apparent K_m of 33.3 μM were calculated. Similar experiments with 10,000 *g* supernatant fractions of the Ben cell line, but with appropriate co-factors for sulphate conjugation, showed that the production of 1-naphthyl sulphate was extremely low and was neither time nor substrate nor enzyme dependent.

Similar experiments with E14 showed no significant metabolism of 1-naphthol to either a glucuronic acid or sulphate ester conjugate.

DISCUSSION

The results summarised in Table 2 show a marked variation in the routes and rates of conjugation in the different cell lines. The variation is to be expected as the cell lines were derived from various types of tumour. However, particularly striking was the finding that none of the cell lines showed the pattern of conjugation using 1-naphthol as substrate observed with normal peripheral human lung, i.e. extensive sulphate ester formation with little or no glucuronic acid conjugation [2]. Thus, the metabolic pathways involved in conjugation were not the same in the cell lines where sulphation was markedly diminished or absent, whilst glucuronic acid conjugation was sometimes increased. The cell line, E14, showed a remark-

Table 2. Metabolism of 1-naphthol and 3-hydroxybenzo(*a*)pyrene (3-OH-BP) by mono-layer cell culture

Cell line	1-Naphthyl-β-D-glucuronide	1-Naphthyl sulphate	3-OH-BP-D-glucuronide	3-OH-BP-hydrogen sulphate
	(nmoles of product formed/mg protein/90 min)			
Ben	16.7	—*	0.20	0.05
Gri	3.9	<0.1	0.02	0.02
Min	6.3	1.1	<0.01	0.02
Poc	<0.1	—	N.D.	N.D.
Mar	0.1	<0.1	<0.01	—
Mor	2.9	1.2	—	0.05
Dre	—	1.0	<0.01	0.03
E14	0.1	0.1	—	<0.01

* Not above control values.

N.D. not determined.

Values in this table are the means of two separate determinations. The two sets of results for each cell line are not true duplicates as the two incubations were carried out with monolayer cell cultures which had grown separately. The figures in this table are not necessarily true rates as the determinations were made after 90 min incubation. These probably represent an underestimate of initial rates particularly with the cell line Ben where almost all the 1-naphhol was metabolised to the glucuronide conjugate.

able lack of conjugating ability compared to the other cell lines. This could be related to the fact that E14 has been in culture for a longer period, producing a loss of metabolic pathways.

The relative lack of sulphate ester formation displayed, using cell cultures of both the E14 and Ben cell lines (Table 2), was also observed using subcellular fractions fortified with appropriate co-factors. This may be due to either the absence of a sulphotransferase or an inability to generate the sulphate donor, adenosine 3′-phosphate 5′-sulphatophosphate (PAPS). Due to the multiplicity of both UDP-glucuronosyltransferase [14] and sulphotransferase enzymes [15], it is necessary to assess conjugation with UDP-glucuronic acid and PAPS using more than one substrate. Initial studies using the human bronchial cancer cell lines with a second substrate, 3-OH-BP, confirmed a relative lack of sulphate ester conjugate formation obtained with 1-naphthol (Table 2). Earlier work from our laboratory has shown that short-term organ cultures of normal peripheral human lung with 3-OH-BP formed the sulphate ester conjugate. However, in that study no attempt was made to ascertain the formation, if any, of the glucuronic acid conjugate [16]. Small but significant amounts of the glucuronic acid conjugate of 3-OH-BP were formed by several of the cell lines, in particular Ben (Table 2). However, compared with 1-naphthol, 3-OH-BP was a much poorer substrate for UDP-glucuronosyltransferase (Table 2) in agreement with the findings of Bock *et al.* [17] using a number of different tissues from the rat. Their studies also suggested that 3-OH-BP and 1-naphthol were conjugated by the same UDP-glucuronosyltransferase [17]. Whilst the results of the present study lend support to this hypothesis, the low activity observed using 3-OH-BP as a substrate precludes a more definite statement.

The previous studies on normal lung tissue [2] with which the results have been compared originate from peripheral lung, but the majority of human lung cancers arise from bronchial epithelium. However, investigations with short-term explant cultures of human bronchus by Autrup *et al.* [18], and initial experiments in our laboratory, show that sulphate ester conjugation is quantitatively more important than glucuronic acid conjugation.

A possible complication in the interpretation of the present studies would arise if there were altered levels of either β-glucuronidase or aryl sulphatase in the tumour cell lines. Elevations of these enzyme activities have been reported in human tumours [19, 20]. Although aryl sulphatase and β-glucuronidase have not been measured in the present study, the overall metabolism by the intact tissue represents a balance of the relative rates of synthesis and hydrolysis of the conjugates.

The results of this study confirm and extend our original observations found when using short-term organ culture of human lung and tumour [3]. Quantitative and qualitative variations in the rates and routes of conjugation were observed dependent, in part, on the tumour cell line. However, none of the cell lines showed the extensive sulphate ester formation which is seen with normal human lung [2]. The decrease in sulphation is accompanied in some cell lines, in particular the poorly differentiated epidermoid carcinoma, by a marked increase in glucuronic acid conjugation. The significance of these changes to the neoplastic process is far from clear, but because of their key roles in the metabolism of drugs, they may be of importance in determining the successful outcome of chemotherapy.

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REFERENCES

1. E. L. Wynder and S. Hecht (Eds), *Lung Cancer*, UICC Technical Report Series, Vol. 25, pp. 3–33. International Union Against Cancer, Geneva (1976).
2. R. Mehta and G. M. Cohen, *Biochem. Pharmac.* **28**, 2479 (1979).
3. G. M. Cohen, E. M. Gibby and R. Mehta, *Nature, Lond.* **291**, 662 (1981).
4. K. K. Lueders, M. M. Dyers, E. B. Thompson and E. L. Kuff *Cancer Res.* **30**, 274 (1970).
5. T. Gessner, *Biochem. Pharmac.* **23**, 1809 (1974).
6. K. W. Bock, F. Lorch and G. Van Ackeren, *Naunyn-Schmiedebergs Archs. Pharmac.* **287** (Suppl.), R77 (1975).
7. A. Winsnes, and M. E. Rystad *Acta. Pharmac. Tox.* **33**, 161 (1973).
8. R. H. Adamson and J. R. Fouts, *Cancer Res.* **21**, 667 (1961).
9. L. A. Rogers, H. P. Morris and J. R. Fouts, *J. Pharmac. exp. Ther.* **157**, 227 (1967).
10. L. G. Hart, R. H. Adamson, H. P. Morris and J. R. Fouts, *J. Pharmac. exp. Ther.* **149**, 7 (1965).
11. M. Ellison, C. J. Hillyard, G. A. Bloomfield, L. H. Rees, R. C. Coombes and A. M. Neville, *Clin. Endocr.* **5** (Suppl.), 397s (1976).
12. R. Mehta, P. C. Hirom and P. Millburn, *Xenobiotica* **8**, 445 (1978).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. G. J. Dutton and B. Burchell in *Progress in Drug Metabolism* (Eds. J. W. Bridges and L. F. Chasseaud), Vol. 2, pp. 1–70. Pergamon Press, Oxford (1976).
15. R. H. De Meio, in *Metabolic Pathways* (Ed. D. M. Greenberg), Vol. 7, pp. 287–385. Academic Press, New York (1975).
16. G. M. Cohen, S. M. Haws, B. P. Moore and J. W. Bridges, *Biochem. Pharmac.* **25**, 2561 (1976).
17. K. W. Bock, U. C. V. Clasbruck, R. Kaufman, W. Lilienblum, F. Oesch, H. Pfeil and K. L. Platt, *Biochem. Pharmac.* **29**, 495 (1980).
18. H. Autrup, F. C. Wefald, A. M. Jeffrey, H. Tate, R. D. Schwartz, B. F. Trump and C. C. Harris, *Int. J. Cancer* **25**, 293 (1980).
19. W. H. Fishman and A. J. Anlyan, *Cancer Res.* **7**, 808 (1947).
20. S. Gasa, A. Makita, T. Kameya, T. Kodoma, E. Araki, T. Yoneyama, M. Hirama and M. Hashimoto, *Cancer Res.* **40**, 3804 (1980).