6. J.-L. Borgna and H. Rochefort, *J. biol. Chem.* **256**, 859 (1981).
7. H. K. Adam, E. J. Douglas and J. V. Kemp, *Biochem. Pharmac.* **27**, 145 (1979).
8. H. K. Adam, J. S. Patterson and J. V. Kemp, *Cancer Treat. Rep.* **64**, 761 (1980).
9. C. P. Daniel, S. J. Gaskell, H. Bishop and R. I. Nicholson, *J. Endocr.* **83**, 401 (1979).
10. A. B. Foster, L. J. Griggs, M. Jarman, J. M. S. van Maanen and H.-R. Schulten, *Biochem. Pharmac.* **29**, 1977 (1980).
11. J. R. Fry and J. W. Bridges, in *Progress in Drug Metabolism* (Eds. J. W. Bridges and L. F. Chassaud), Vol. 2, p. 71. Wiley, New York (1977).
12. D. J. Bates, A. B. Foster and M. Jarman, *Biochem. Pharmac.* **30**, 3055 (1981).
13. V. C. Jordan, L. Rowsby, C. J. Dix and G. Prestwich, *J. Endocr.* **78**, 71 (1978).
14. V. C. Jordan and K. E. Allen, *Eur. J. Cancer* **16**, 239 (1980).
15. M. Sluyser, S. G. Evers and C. C. J. de Goeij, *Eur. J. Cancer clin. Oncol.* **17**, 1063 (1981).
16. M. E. Lippman, G. Bolan and K. Huff, *Cancer Res.* **36**, 4595 (1976).
17. G. Leclercq, N. Devleeschouwer, N. Legros and J. C. Heuson, in *Cytotoxic Estrogens in Hormone Receptive Tumours* (Eds. J. Raus, H. Martens and G. Leclercq), p. 165. Academic Press, New York (1980).
18. V. C. Jordan, M. M. Collins, L. Rowsby and G. Prestwick, *J. Endocr.* **75**, 305 (1977).
19. D. W. Mendenhall, H. Kobayashi, F. M. L. Shih, L. A. Sternson, T. Higuchi and C. Fabian, *Clin. Chem.* **24**, 1518 (1978).
20. G. Leclercq, M. C. Deboel and J. C. Heuson, *Int. J. Cancer* **18**, 750 (1976).
21. K. Burton, *Biochem. J.* **62** 315 (1956).
22. C. R. Hicks, *Fundamental Concepts in the Design of Experiments*, p. 30. Holt, Rinehart and Winston, New York (1973).

Biochemical Pharmacology, Vol. 31, No. 17, pp. 2827–2830, 1982.
Printed in Great Britain.

0006-2952/82/172827-04 $03.00/0

Diuretic action of BTS 39542 (dihydrophthalazin-1-ylacetic acid) is consistent with inhibition of Na + K + Cl cotransport

(*Received* 5 *February* 1982; *accepted* 29 *March* 1982)

BTS 39542 (dihydrophthalazin-1-ylacetic acid) is a potent diuretic agent in rats, rabbits and dogs [1] which is structurally unrelated to other high-efficacy diuretics [1]. The exact renal tubular locus and mechanism of action of BTS 39542 are, however, unknown. Simple pharmacological model systems of diuretic action such as anuran skin [2] or human red cells [3] have been identified whose major ion transport systems reflect the functionally important ion transport of microdissected, microperfused thick ascending limb and distal renal tubules [3, 4]. In the present paper we have attempted to characterise the molecular action of BTS 39542 by comparing its effect with loop diuretics such as furosemide upon the passive Na + K + Cl contransport system in cultured human carcinoma cells (HeLa), cultured canine renal epithelial cells (MDCK) and human red blood cells.

Materials and methods

Human red cells were obtained by venepuncture from healthy volunteers. After washing four times in a medium containing 150 mM NaCl, 10 mM Tris–HCl, 6.0 mM KCl, 10 mM glucose, pH 7.4 and removal of the buffy coat, red cells were used for ^{86}Rb influx measurements at approximately 70% haematocrit.

HeLa cells were grown in Eagle's Basal Medium supplemented with 10% new-born calf serum [5]. For experimental purposes, monolayers of cells were grown to confluency in 60 mm Sterilin plastic petri dishes (approximately 2×10^6 cells/plate).

MDCK cells form monolayers possessing epithelial structure and capable of transmonolayer ion transport [6]. Cultures of MDCK cells were obtained at 60 serial passages from Flow Laboratories (Irvine, U.K.) and maintained in serial culture in Roux flasks in Minimum Essential Eagles (M.E.M.E.) supplemented with non-essential amino acids, (2 mM glutamine, 1 unit/cm^3 gentamycin antibiotic and 10% v/v foetal bovine serum (Flow)) at 37° in an air–5% CO_2 atmosphere. For experiments, cells were grown in 60 mM Sterilin plastic petri dishes to form subconfluent cell monolayers.

K influx measurements were made using ^{86}Rb as a tracer in all experiments [5, 7, 8]. Influx determinations (30 min) were made in human red cells at 37° by a slight modification of the method of Lauf and Joiner [9] using centrifugation through dibutylphthalate to separate cells from the radioactive influx solution. For cultured cells, the ^{86}Rb influx into cell monolayers was measured at 37° during a 10 min (HeLa) or 5 min (MDCK) incubation in Krebs' solution containing 137 mM NaCl; 5.4 mM KCl; 2.8 mM $CaCl_2$; 1.2 mM $MgSO_4 \cdot 7H_2O$; 0.3 mM NaH_2PO_4; 0.3 mM KH_2PO_4; 12 mM HCl; 14 mM Tris base and 11 mM glucose supplemented with 1.0% v/v new-born calf serum, pH 7.4 and 0.2 μCi/ml ^{86}Rb. At the end of the influx period, the monolayer was rapidly rinsed four times with ice-cold Krebs' solution to remove extracellular isotope and then trypsinised to form a single cell suspension. After trypsinisation, the cell number and mean cell volume of each experimental plate was determined on a Coulter Counter (Model ZF) fitted with a Channelyser attachment (Model C1000). The ^{86}Rb content of all samples was measured in a liquid scintillation spectrophotometer (Packard, model 3255) by the Cerenkov method.

The effect of chronic BTS 39542 treatment upon Na–K pump function was determined in HeLa cells by measuring specific [^{3}H]ouabain binding to determine Na^+ pump number [5] from a media containing 2×10^{-7} M ouabain in K-free Krebs solution or one containing 15 mM KCl for 20 min [5]. At the end of the incubation period the cells were rinsed four times with ice-cold Krebs solution and the cell number, mean cell volume determined, as well as the ^3H content. Intracellular Na + K contents were also measured by flame photometry after rinsing cell layers four times with ice-cold choline chloride followed by a 3-hr incubation in deionised water. Cell number and cell volume were determined on parallel samples as above.

Table 1. Effects of BTS 39542 (10^{-4} M) and furosemide (10^{-4} M) upon ^{86}Rb(K) influx in HeLa, MDCK and red blood cells.

Cell type	n	^{86}Rb influx Control	Ouabain (10^{-3} M)	Furosemide (10^{-4} M)	Furosemide (10^{-4} M) +ouabain (10^{-3} M)	BTS 39542 (10^{-4} M)	BTS 39542 (10^{-4} M) +ouabain (10^{-3} M)	Diuretic sensitive flux (−ouabain)	Diuretic sensitive flux (+ouabain)
HeLa (mmoles/l cell water/min)	(4)	4.20 ± 0.20	2.48 ± 0.10§	2.59 ± 0.10§	0.41 ± 0.01§	2.72 ± 0.10§	0.30 ± 0.01§	1.62 ± 0.31* NS 1.48 ± 0.27†	2.07 ± 0.12* NS 2.19 ± 0.11†
MDCK (mmoles/l cell water/min)	(5)	7.34 ± 0.31	5.26 ± 0.51‡	3.83 ± 0.13§	0.70 ± 0.02§	3.77 ± 0.24§	0.58 ± 0.06§	3.51 ± 0.30* NS 3.57 ± 0.43†	4.56 ± 0.52* NS 4.68 ± 0.59†
Human red blood cells (mmoles/l packed cells/hr)	(4)	2.46 ± 0.03	1.38 ± 0.02§	1.46 ± 0.03§	0.22 ± 0.03§	1.53 ± 0.04§	0.11 ± 0.02	1.01 ± 0.06* NS 0.92 ± 0.07†	1.18 ± 0.03* NS 1.28 ± 0.04†

Values are the mean ± S.E.M.
* diuretic sensitive flux due to furosemide.
† diuretic sensitive flux due to BTS 39542.
Significantly different from control values (Student's t test): ‡ $P < 0.01$, § $P < 0.001$.
Significantly different from furosemide sensitive flux: N.S. not significant.

^{86}Rb was purchased from the Radiochemical Centre (Amersham, U.K.). Tissue culture supplies were obtained from Flow Laboratories (Irvine, U.K.) and Gibco-Biocult Ltd. (Paisley, U.K.).

BTS 39542 was a gift from Dr. M. Sim, Boots Co Ltd. (Nottingham, U.K.) and stock solutions were made in 10^{-2} Tris. Other materials were obtained as stated previously [7].

Variation in results is expressed as the standard error of the mean. Tests for significance of differences were made by a two tailed Student's *t*-test (unpaired means solution).

Results and discussion

Potassium influx into human red cells [3], HeLa [7] and MDCK [7, 8] cells may be separated into three components, namely a ouabain sensitive component consisting of Na^+,K^+-ATPase mediated transport (Table 1, columns 3–4), a ouabain insensitive but furosemide sensitive component mediated via a Na + K + Cl cotransport system (Table 1, columns 9–10) and a ouabain and furosemide insensitive component representing the ground permeability of the membrane to K^+ (Table 1, column 6) (see also Refs. 10 and 11). The furosemide-sensitive component of K^+ influx is of particular significance since this diuretic-sensitive transport system may give rise to secondary active Cl^- transport using the energy from K or Na gradients [3, 12]. Thus, in mammalian thick ascending limb the major features of the furosemide senstive transport located upon the apical membrane strongly resemble the known features of the furosemide sensitive cotransport system in MDCK, HeLa, red cell and ascites cells [7, 10, 13].

BTS 39542 at 10^{-4} M causes a significant reduction in K^+ influx in both the presence and absence of 10^{-3} M ouabain in HeLa, MDCK and human red blood cells (Table 1). The diuretic sensitive flux for all 3 cell types in the presence or absence of ouabain is similar, whether furosemide or BTS 39542 is used; moreover, BTS 39542 together with furosemide (10^{-4} M) does not produce an additive reduction in K^+ influx (e.g. for MDCK cells, furosemide plus BTS 39542 treated cells, the K^+ influx is 3.33 ± 0.15 mmoles/l cell water/min compared to 3.83 ± 0.13 or 3.77 ± 0.24 mmoles/l cell water/min for furosemide, or BTS 39542 treated cells). The ouabain insensitive but diuretic sensitive K^+ flux shows a marked dependence on medium Na^+ and Cl^- consistent with the coupled ($Na^+ + K^- + Cl^-$) nature of this transport process; with inhibition of the cotransport system by replacement of medium Cl by NO_3 no BTS 39542 sensitive component is observed (e.g. in red cells the ouabain-insensitive K^+ influx in NO_3^- media is 0.16 ± 0.01 ($n = 4$) mmole/l packed cells/hr, with BTS 39542 (10^{-4} M) this flux is unchanged at 0.18 ± 0.04 ($n = 4$) mmole/l packed cells/hr. $P > 0.5$, N.S.).

This data is therefore consistent with BTS 39542 inhibition of the Na + K + Cl cotransport system; an effect of BTS 39542 on active ion transport via the Na^+, K^+)-ATPase is unlikely since the effects of BTS 39542 upon K^+ influx are approximately additive with ouabain (Table 1). The small difference between diuretic sensitive fluxes in the presence and absence of furosemide observed with furosemide or BTS 39542 (Table 1) is most likely due to an interdependence of pump/leak pathways rather than a direct effect of the diuretics on Na–K pump function. A test of alteration in Na–K pump function was made by chronic (24 hr) applications of BTS 39542 (10^{-4} M) in the growth medium of HeLa cells [5]; BTS 39542 neither markedly reduced specific [^{3}H]ouabain binding to intact HeLa cells (control binding = 0.60 ± 0.02 (S.E.) $\times 10^6$ sites/cell, chronic BTS 39542 treatment = 0.73 ± 0.02 (S.E.) ($n = 5$) $\times 10^6$ sites/cell) or more significantly, raised intracellular Na contents (control Na and K contents = 14 ± 1, 209 ± 2 (S.E.) ($n = 5$) mmoles/l cell water respectively, chronic BTS 39542 treatment = 14 ± 1, 228 ± 3 (S.E.) ($n = 5$) mmoles/l cell water respectively, $P > 0.9$, $P < 0.01$ for increases in Na and K contents respectively).

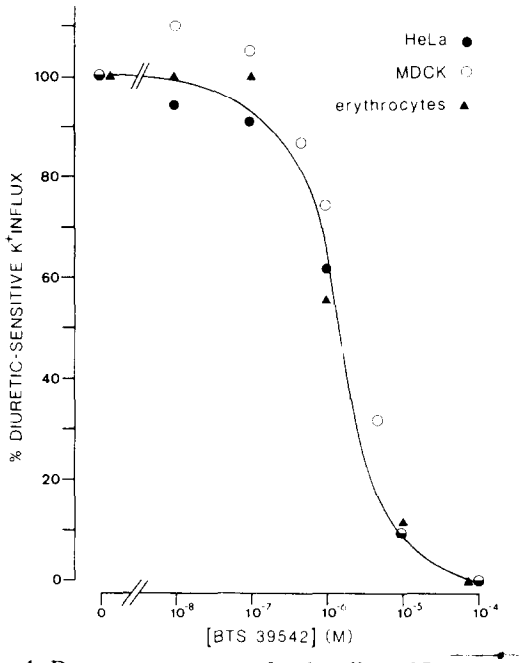

Fig. 1. Dose–response curves for the effect of BTS 39542 upon the ouabain insensitive ^{86}Rb influxes in human red blood cells (▲), HeLa (●) and MDCK cells (○). Each point is the mean of at least 4 determinations. Standard errors for all points are < 5%. For human red cells ^{86}Rb influxes in the presence and absence of 10^{-3} M ouabain were 2.5 ± 0.1 ($n = 4$) and 0.74 ± 0.02 ($n = 4$) mmole/l packed red cells/hr. For HeLa and MDCK cells these fluxes were 4.4 ± 0.2 ($n = 4$), 2.5 ± 0.1 ($n = 4$) and 11.67 ± 0.62 ($n = 4$), 7.99 ± 0.29 ($n = 4$) mmoles/l cell water/min, respectively. The diuretic-insensitive flux in the presence of ouabain is subtracted from all results.

Figure 1 shows the dose-dependency of BTS 39542 action upon the ouabain insensitive fluxes in HeLa, MDCK, and human red cells; 50% inhibition determined using probit analysis is observed at 1.0 ± 0.2 (S.D.) $\times 10^{-6}$ M, 2.2 ± 0.2 (S.D.) $\times 10^{-6}$ M and 1.8 ± 0.4 (S.D.) $\times 10^{-6}$ M in the three cell systems respectively, these values compare with 5×10^{-6} [7], $2–3 \times 10^{-6}$ [8] and 9.0×10^{-6} M [3] for furosemide inhibition of ouabain insensitive K^+ influxes in HeLa, MDCK and red cells, respectively. The similarities between the inhibitory potency of BTS 39542 and furosemide in dog cells reported here differs from their relative diuretic potencies in dogs and this is suggestive of more than a single tubular locus for BTS 39542 action or extra renal actions [1].

We have tested BTS 39542 in the isolated frog-skin preparation, but no inhibition of the short-circuit current with BTS 39542 (10^{-4} M) was observed although amiloride (10^{-4} M) gave a 96% reduction in the short-circuit current (unpublished data). The major site of BTS 39542 action, consistent with present data, is thus the Na + K + Cl cotransport system in the thick ascending limb which gives rise to secondary active Cl^- movement [3, 13]. Finally, it is pertinent to note that the novel structure of BTS 39542 compared to furosemide derivatives [3] should be of use in defining structure–inhibition relationships for the cotransport system.

Acknowledgements—We are grateful to the National Kidney Research Fund, The Wellcome Trust and The Royal Society Equipment Fund for financial support. Miss Carol Walker gave skilled technical assistance.

Department of Physiology and Pharmacology
University of St. Andrews
St. Andrews
Fife KY16 9TS, U.K.

JAMES F. AITON
NICHOLAS L. SIMMONS

REFERENCES

1. M. J. Cooling and M. F. Sim, *Br. J. Pharmac.* **74**, 359 (1981).
2. A. W. Cuthbert and W. K. Shum, *Molec. Pharmac.* **10**, 880 (1974).
3. J. C. Ellory and G. W. Stewart, *Br. J. Pharmac.* **75**, 183 (1982).
4. J. A. Schafer and T. E. Andreoli, in *Membrane Transport in Biology* (Ed. G. Giebisch), Vol. IVB, p. 473. Springer, New York (1979).
5. J. F. Aiton, J. F. Lamb and P. Ogden, *Br. J. Pharmac.* **73**, 333 (1981).
6. N. L. Simmons, *J. membr. Biol.* **59**, 105 (1981).
7. J. F. Aiton, A. R. Chipperfield, J. F. Lamb, P. Ogden and N. L. Simmons, *Biochim. biophys. Acta* **646**, 389 (1981).
8. J. F. Aiton, C. D. A. Brown, P. Ogden and N. L. Simmons, *J. membr. Biol.* **65**, 99 (1982).
9. P. K. Lauf and C. H. Joiner, *Blood* **48**, 457 (1976).
10. P. Geck, E. Heinz, C. Pietrzyk, B. Pfeiffer, in *Cell Membrane Receptors for Drugs and Hormones* (Ed. R. W. Straub and I. Bolis), p. 301. Raven Press, New York (1978).
11. P. Geck, C. Pietrzyk, B. C. Burckhardt, B. Pfeiffer and E. Heinz, *Biochim. biophys. Acta* **600**, 432 (1980).
12. C. D. A. Brown and N. L. Simmons, *Biochim. biophys. Acta* **649**, 427 (1981).
13. R. Greger, *Pflügers Arch. ges. Physiol.* **390**, 38 (1981).

Biochemical Pharmacology, Vol. 31, No. 17, pp. 2830–2833, 1982.
Printed in Great Britain.

0006-2952/82/172830-04 $03.00/0

2,3,4,4′,5-Pentachlorobiphenyl: differential effects on C57BL/6J and DBA/2J inbred mice

(*Received* 23 *November* 1981; *accepted* 26 *February* 1982)

Polychlorinated biphenyls (PCBs) typify a group of halogenated aromatic chemicals which include the polychlorinated dibenzofurans (PCDFs), dibenzo-*p*-dioxins (PCDDs), naphthalenes and polybrominated biphenyls (PBBs). These chemicals have a number of common chemical and biological properties [1], e.g. (1) within each group there exists a multiplicity of isomers and congeners, (2) there are dramatic effects of structure on the biologic and toxic potencies of individual halogenated aromatic compounds, and (3) like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic individual halogenated aromatic compound, a limited number of approximate stereoisomers elicit comparable toxic responses [2–9]. Included in this latter group are 3,3′,4,4′,5,5′-hexachlorobiphenyl (HCBP) and hexabromobiphenyl (HBBP) [2, 9]. In addition, it has been shown that the 2,3,7,8-TCDD and related compounds induce the microsomal cytochrome P-450-dependent monooxygenase, benzo[*a*]pyrene hydroxylase (or aryl hydrocarbon hydroxylase, AHH) and reversibly bind to a high-affinity cytosolic receptor protein [3, 10, 11]. The non-covalently bound ligand (inducer)–receptor complex is transported into the nucleus and subsequently controls the expression of AHH induction and related toxic responses [9–13]. The cytosolic receptor protein, a product of the *Ah* locus in mice, is found in higher concentrations in certain mouse strains, such as the C57BL/6J inbred strain [12, 13], which are highly sensitive to the biologic and toxic effects of 2,3,7,8-TCDD, 3,3′,4,4′,5,5′-HCBP, 3,3′,4,4′,5,5′-HBBP and related compounds [2, 9, 14]. In contrast, the DBA/2J mouse strain contains lower levels of the high-affinity receptor protein and is much less susceptible to the inductive and toxic effects of 2,3,7,8-TCDD and related compounds [9, 14].

Recent reports have described a group of PCB and PBB isomers and congeners that induce a pattern of hepatic microsomal enzyme activities consistent with the coadministration of 3-methylcholanthrene (3-MC, an AHH inducer) plus phenobarbitone (PB) [8, 15–19]. Some of these mixed-type inducers are toxic to chick embryos [20] and rats [21]; however, their toxicities and activities as AHH inducers in inbred mouse strains have not been determined. This study reports the biologic and toxic effects elicited by 2,3,4,4′,5-pentachlorobiphenyl (PCBP), a mixed-type inducer in rats, in the responsive C57BL/6J and the non-responsive DBA/2J mice.

PCBP and 2,2′,4,4′,5,5′-HCBP were > 99% pure and prepared as described [15]. The synthetic route and purification procedures were developed to preclude the presence of PCDD and PCDF contaminants. Ascorbic acid and phenazine ethosulfate were purchased from the Sigma Chemical Co., St. Louis, MO. The sources of other chemicals used in this study are described elsewhere [15]. Adult C57BL/6J and DBA/2J inbred strains of mice were obtained from the Jackson Laboratory, Bar Harbor, ME. The mice were housed in wire cages, maintained on a 12-hr diurnal light regimen, and allowed free access to Purina Certified Rodent Chow (No. 5002) and water. Both strains of mice were injected intraperitoneally with PB or 3-MC (400 μmoles/kg body wt) on three consecutive days, and the animals were killed by cervical dislocation 24 hr after the last injection. The C57BL/6J mice were injected with two doses of PCBP (either 150 or 750 μmoles/kg) on days 1 and 3 and killed on day 6. DBA/2J mice received only the high dose of PCBP. Mice injected with corn oil (20 ml/kg) serves as controls. All animals were fasted over the last 24 hr to lower liver glycogen levels. The thymic involution studies were carried out as described above except that the animals were not fasted over the last 24 hr.

The mouse livers were cleared of blood by perfusing via the hepatic portal vein with ice-cold isotonic saline containing EDTA (100 μM), and the liver weights were determined. Thymuses were excised, trimmed, blotted dry, and weighed. The liver microsomal fraction was isolated as a 100,000 *g* pellet by further centrifugation of a 10,000 *g* supernatant fraction from the whole liver homogenate [8].

The concentration of cytochrome P-450 was determined