

## **Ionic Contra-Viral Therapy (ICVT); a new approach to the treatment of DNA virus infections**

### Brief Report

**C. Hartley, M. Hartley, I. Pardoe, and A. Knight**

Henderson Morley Plc, Moseley, Birmingham, U.K.

Received May 9, 2006; accepted June 20, 2006  
Published online August 21, 2006 © Springer-Verlag 2006

**Summary.** The sequestration of cellular  $K^+$  has been shown elsewhere to elicit a broad spectrum of antiviral activity. The obligatory, coupled cotransports of  $Na^+$ ,  $K^+$  and  $Cl^-$  (NKCC1) and of  $Na^+$  and  $K^+$  (NKATPase) effect net cellular  $K^+$  influx. We examined the effects of specific inhibitors of these transports; a cardiac glycoside (Digoxin) and a loop diuretic (Furosemide) on virus replication *in vitro*. The replication of the DNA viruses, herpes simplex virus, varicella zoster virus, human cytomegalovirus and adenovirus was inhibited. There was normal replication of the RNA virus encephalomyocarditis virus. Antiviral activities of both drugs were influenced by extracellular  $K^+$ . Antiviral effects were most potent when Digoxin and Furosemide were used in combination. Targeting the host cell in this way is fundamentally different to other antiviral drug developments to date and we propose the descriptive term Ionic Contra Viral Therapy (ICVT) for the purpose of definition. We believe that specific inhibitors of coupled  $K^+$  transports merit controlled clinical trial for a broad spectrum of DNA virus infections by local application.

\*

The obligatory, coupled cotransport of  $Na^+$ ,  $K^+$  and  $Cl^-$  by cell membranes has been reported in nearly every animal cell type. Two isoforms of the  $Na^+$ ,  $K^+$  and  $Cl^-$  cotransporter (NKCC) protein are currently known; NKCC2 is found exclusively in the kidney and NKCC1 is found in nearly all cell types. Their functions include the maintenance of a higher internal  $Cl^-$  concentration than the predicted electrochemical equilibrium and the regulation of cell volume. The history of its discovery and its fundamental properties have recently been comprehensively reviewed and extensively referenced [21].

Sodium-potassium ATPase (NKATPase) is another obligatory, coupled co-transporter also found in almost all animal cells. It is important in the maintenance of  $\text{Na}^+$  and  $\text{K}^+$  gradients across the plasma membrane and fundamental in providing energy for several essential cellular functions including the control of membrane potential and cell volume [22].

While the NKCCs are responsible for a net influx of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$ , NKATPase effects net influx of  $\text{K}^+$  and  $\text{Na}^+$  efflux. This study is primarily concerned with  $\text{K}^+$  influx since it has been shown by X-ray microanalysis that  $\text{K}^+$  is an essential factor in viral DNA synthesis [8].

Viruses are obligate, intracellular parasites and while the viral genome usually encodes macromolecules with functions specific and vital to the virus, it is ultimately dependent upon the milieu of the infected host cell for its proliferation. Curiously, human cytomegalovirus (HCMV) has been shown to subvert the cell's electrodynamics to its advantage; during the normal course of infection, NKCC1 is translocated from the plasmalemma to a perinuclear site, halting the cell cycle in the G1/S interphase and accommodating the characteristically long life cycle of HCMV [16].

$\text{Li}^+$  inhibits replication of herpes simplex virus (HSV) and other DNA viruses *in vitro* [2, 20, 23, 29]. It was subsequently shown that  $\text{Li}^+$  mediated the sequestration of intracellular  $\text{K}^+$  leading to inhibition of viral DNA synthesis. In addition, culture in potassium-depleted tissue culture medium, without recourse to  $\text{Li}^+$ , inhibited HSV DNA synthesis just as effectively [8]. Under these conditions, viral gene products necessary for HSV DNA synthesis, for example thymidine kinase and DNA polymerase, were available in abundance, yet the synthesis of progeny viral DNA, which is essential to virus proliferation and infectivity, was completely inhibited.

Antiviral drug development is guided, in part, by the need to minimise adverse consequence to the host and it has tended to focus, therefore, on synthetic pathways peculiar to the particular virus under consideration and distinct from those of the host cell. For example, acyclic nucleoside analogues, which can be incorporated into nascent DNA molecules, preventing chain extension [24], and antisense oligonucleotides, which are short, synthetic DNA molecules designed to inhibit translation of a targeted gene via interaction with messenger RNA [1]. Both types of molecule are effective through intimate interaction with virus-specific biosynthetic pathways and their antiviral activities are usually extremely virus specific.

In the absence of effective chemotherapies, viral infections continue to have widespread adverse impact: Adenoviruses (AV), for example, are the most common cause of acute red eye; vision may be impaired during the acute phase of infection and in severe cases, immune complexes deposit in the cornea, leading sometimes to permanent visual impairment. The human herpesvirus varicella zoster virus (VZV) causes corneal disease and acute retinal necrosis, and human cytomegalovirus (HCMV) causes retinitis, often leading to permanent visual impairment [4, 12].

## Ionic Contra-Viral Therapy for treating DNA virus infections

If, as we believe, different DNA viruses are dependent upon  $K^+$  for replication, then the controlled depletion of cellular  $K^+$  might elicit a broad spectrum of antiviral activity.

The inhibitory effects of cardiac glycosides and loop diuretics on coupled cotransporters are well known. Digoxin, for example, inhibits NKATPase [6] while the loop diuretic Furosemide inhibits NKCC1 [7]; both drugs inhibiting  $K^+$  influx. We report here the effects of these drugs on the replication of a broad spectrum of DNA viruses, namely adenovirus (AV) and the human herpesviruses varicella zoster virus (VZV), cytomegalovirus (HCMV) and herpes simplex virus (HSV), as well as an RNA virus, encephalomyocarditis virus (EMCV).

Furosemide injection BP was obtained from Antigen Pharmaceuticals and Digoxin injection BP from the Glaxo Wellcome Group of Companies. Cells were propagated in Eagle's medium supplemented with non essential amino acids, L-glutamine and 10% (v/v) foetal bovine serum (FBS), 10,000 units/ml penicillin and 10,000  $\mu$ g/ml streptomycin and maintained in medium supplemented with 2% FBS. MTT assays were performed as described elsewhere [19].

Plaque reduction assays (PRA) were used to determine antivirally effective drug concentrations. The 50% plaque inhibitory concentrations (IC<sub>50</sub>), based on three separate determinations, were taken from best-fit plots and presented as mean  $\pm$  SD (Table 1). The nature of drug interaction was evaluated using the formula:

$$1 = D1/ID_{x1} + D2/ID_{x2}$$

where  $ID_{x1}$  and  $ID_{x2}$  are the concentrations of each respective drug alone, and D1 and D2 are the concentrations of each drug in the mixture that yield 50% inhibition. When the right hand side (RHS) of the equation is less than 1.0, then Loewe synergism is indicated, when greater than 1.0, then Loewe antagonism is indicated [14].

The effects of Digoxin and Furosemide on virus replication and on the rate of cell metabolism are summarised in Table 1, and the following observations will be considered later in discussion: 1. In MRC5 cells, the Digoxin and Furosemide IC<sub>50</sub>s for HSV2 (186), HCMV (AD169) and VZV (Ellen) were virtually identical. 2. In A549 cells, the Digoxin and Furosemide IC<sub>50</sub>s for AV Usha (serotype 10) and AV McEwan (serotype 5) were indistinguishable. 3. In Vero cells, both the Digoxin IC<sub>50</sub>s and the Furosemide IC<sub>50</sub>s were significantly different for HSV2 (186) and HSV1 (McKrae). 4. In BHK 21 cells, there was normal replication of the RNA virus, EMCV, in the presence of either Digoxin (60 ng/ml) or Furosemide (1 mg/ml) at concentrations inhibitory to HSV replication.

Neither Digoxin (500 ng per ml) nor Furosemide (1 mg per ml) neutralized virus infectivity as adjudged by incubation of cell-free virus suspension in drug containing media for 24 h at +4 °C, and the excipients of intravenous preparations were antivirally inactive at the concentrations concerned here, as adjudged by appropriate control PRAs.

**Table 1.** The effects of Digoxin and Furosemide on virus plaque formation and cell metabolism (MTT assay)

Virus	Host cell	Furosemide IC50 ( $\mu\text{g/ml}$ )	MTT reduction	Digoxin IC50 (ng/ml)	MTT reduction
HSV2 (186)	MRC5	460 $\pm$ 114	10% (48 hrs)	20 $\pm$ 7.5	20% (48 hrs)
HSV2 (186)	VERO	1000 $\pm$ 138	10% (48 hrs)	30 $\pm$ 8.0	20% (48 hrs)
HSV2 (186)	BHK21	800 $\pm$ 200	NT	30 $\pm$ 10	NT
HSV1 (McKrae)	VERO	2000 $\pm$ 200	30% (48 hrs)	60 $\pm$ 21	20% (48 hrs)
CMV (AD169)	MRC5	430 $\pm$ 120	10% (7 days)	20 $\pm$ 7.5	30% (7 days)
VZV (Ellen)	MRC5	470 $\pm$ 106	10% (7 days)	20 $\pm$ 7.5	30% (7 days)
AV (Usha)	A459	440 $\pm$ 114	10% (7 days)	77 $\pm$ 22	20% (7 days)
AV (McEwen)	A459	500 $\pm$ 122	10% (7 days)	60 $\pm$ 25	20% (7 days)
EMCV	BHK21	Neg 1mg/ml	20% (5 days)	Neg 60	20% (5 days)

50% plaque inhibitory concentrations ( $\text{IC}_{50} \pm 2 \times \text{SEM}$ ) for Furosemide and Digoxin against the DNA viruses herpes simplex virus (*HSV*), cytomegalovirus (*CMV*), varicella zoster virus (*VZV*) and adenovirus (*AV*), and the RNA virus encephalomyocarditis virus (*EMCV*). The percentage inhibition of the rates of uninfected cell metabolism are given together with the duration of drug exposure; the same time period required by the corresponding plaque reduction assays

MRC-5 cells [10] and BHK-21 cells [15] were obtained from BioWhittaker. Vero cells were a gift from The Doheny Eye Institute, University of Southern California USA. A549 cells [5] were obtained from ATCC. Cells were propagated in Eagle's medium supplemented with non essential amino acids, L-glutamine and 10% (v/v) foetal bovine serum (FBS), 10,000 units/ml penicillin and 10,000  $\mu\text{g/ml}$  streptomycin and maintained in medium supplemented with 2% FBS

Strain 186 of HSV2 was obtained from PHLS, Colindale, UK. The Ellen strain of VZV, the AD169 strain of HCMV and encephalomyocarditis virus (*EMCV*) were obtained from ATCC. Adenovirus (*AV*) serotype 10 (Usha) was isolated from an ocular infection and *AV* serotype 5 (strain McEwen) and HSV1 (McKrae) were gifts from The Doheny Eye Institute. The rate of uninfected cell metabolism was reduced by only 20–30% by Digoxin or Furosemide at their  $\text{IC}_{50}$ s and uninfected cell morphology was normal. Uninfected cell replication, however, was inhibited by Digoxin or Furosemide at their respective viral  $\text{IC}_{50}$

PRA were undertaken using HSV 2(186) in Vero cells in media containing supplemental  $\text{K}^+$ . While normal virus replication was observed in Digoxin- (30 ng per ml) treated cells supplemented with 20 mM  $\text{K}^+$ , the Furosemide  $\text{IC}_{50}$  was reduced (500  $\mu\text{g}$  per ml from 1000  $\mu\text{g}$  per ml) in cells supplemented with 20 mM  $\text{K}^+$ .

When applied in combination at fractional  $\text{IC}_{50}$  concentrations, there was enhanced antiviral activity. *AV* replication was inhibited by 50% when both drugs were applied at  $1/4 \times$  individual  $\text{IC}_{50}$  concentrations, i.e. Digoxin 20 ng/ml and Furosemide 100  $\mu\text{g}$  per ml, indicating Loewe synergy ( $\text{RHS} = 0.5$ ). The rate of cell metabolism was decreased by 20–25%.

HCMV replication was inhibited by 50% when both drugs were applied at  $1/3 \times$  individual  $\text{IC}_{50}$  concentrations, i.e. 7 ng/ml and 130  $\mu\text{g/ml}$ , respectively, indicating Loewe synergy ( $\text{RHS} = 0.6$ ). The rate of cell metabolism was decreased by 20–25%.

## Ionic Contra-Viral Therapy for treating DNA virus infections

Digoxin and Furosemide interact specifically with the cell membrane ion cotransporters NKATPase [6] and NKCC1 [7], respectively. Targeting the host cell in this way is fundamentally different to other antiviral drug developments to date, and we propose the descriptive term Ionic Contra Viral Therapy (ICVT) for the purpose of definition.

It has been reported elsewhere that inhibition of ATP-sensitive potassium channels reversibly arrested cells in the G0/G1 phase of the cell cycle, resulting in the inhibition of cell proliferation [28], and it is consistent that we found inhibited cell replication. However, the rate of uninfected host cell metabolism was only moderately decreased (10–30%) by Digoxin and Furosemide at their virus IC50, and otherwise ‘normal’ cell function was exemplified by the normal replication of the RNA virus, EMCV, in the presence of Digoxin or Furosemide at concentrations inhibitory to DNA virus replication.

Within a single host cell type, either MRC5 or A549, both the Digoxin and Furosemide virus IC50s were the same for different viruses, suggesting that antivirally effective drug concentrations are a function of the drug affinities for, and the relative abundances of, NKATPase and NKCC1 sites within the membranes of the different host cell types.

However, both the Digoxin IC50s for HSV2 (186) and HSV1 (McKrae) and the Furosemide IC50s, in Vero cells were significantly different, suggesting perhaps that different viruses carry subtly different ionic requirement. Indeed, the sensitivities to different antiherpetic compounds of a selection of different isolates of HSV have been studied, and various cross-sensitivity and resistance patterns to various other antiviral drugs have been demonstrated [18]. It is, of course, possible that laboratory strains and clinical isolates might exhibit different IC50s.

Supplemental extracellular K<sup>+</sup> restored virus replication in Digoxin-treated cells, presumably through competitive inhibition of digoxin. However, the virus IC50 of Furosemide was decreased by supplemental K<sup>+</sup>. At first surprising, this is consistent with the much earlier finding that there was “apparent synergistic action of Furosemide and external Rb<sup>+</sup> on K<sup>+</sup> efflux” [11].

In our study, neither Digoxin nor Furosemide was inhibitory to the replication of the RNA virus EMCV. Ulug et al. [27], however, noted changes in Na<sup>+</sup>–K<sup>+</sup> ATPase activity during the normal course of Sindbis virus (an RNA virus) infection and Nagai et al. [17] attributed the inhibition of replication of another RNA virus, namely Sendai virus, by another inhibitor of NKATPase, namely ouabain, to depletion of cellular potassium. Link et al. [13], however, attributed the inhibition of cytopathology of another RNA virus, namely, Newcastle disease virus, by ouabain to “stearic hindrance of virus attachment”. Inhibitors of ion cotransporters clearly have impact upon the replication of some RNA viruses, though the mechanisms remain unclear. Finally, it has been reported that there were reduced levels of human immunodeficiency virus (a retrovirus) in cells cultured in potassium-depleted medium [3]. We are unaware of other, similar studies undertaken with DNA viruses.

The replication of AV and HCMV was most effectively inhibited by the simultaneous application of Digoxin and Furosemide, fulfilling Loewe’s [14]

requirements for synergy. Given the complexities of the interactions of ion transporters, their peculiar ionic dependencies and their sometimes biphasic regulation by extracellular ion concentrations [22] as well as the recently recognised influence of viruses upon them [16], it is difficult at this stage to be more precise than to suggest that the contraviral synergy reported here represents a function of transporter expression, their relative abundances, their drug affinities and the net consequence upon cellular ion distributions.

Human cytomegalovirus (HCMV) was recently shown to effect a translocation of NKCC1 from the plasma membrane to a perinuclear site, thereby preventing cell replication and accommodating the characteristically long life cycle of HCMV [16]. The authors postulated a down-regulation of the NKCC cotransporter protein that might “subserve the need of the virus to halt the cell cycle at the G1/S phase”. Since the translocated NKCC1 is thought to be inactive, we might argue that Furosemide inhibits HCMV replication at an event preceding that of the translocation and inactivation of NKCC1 since we believe, by extrapolation of more detailed work concerning the role of  $K^+$  in HSV replication [8], that Furosemide is effective by inhibiting HCMV DNA synthesis. It seems likely, therefore, that the HCMV-mediated translocation of NKCC must be dependent upon the prior synthesis of HCMV DNA. Indeed, HCMV DNA synthesis peaks 18–24 hrs and 60–80 hrs after infection and the perinuclear accumulation of NKCC appears 72 hrs after infection [25, 26].

## References

1. Agrawal S (1992) Antisense oligonucleotides as antiviral agents. *Trends Biotechnol* 10: 152–158
2. Cernescu C, Popescu L, Constantinescu S (1988) Antiviral effect of lithium chloride. *Virologie* 39: 93–101
3. Choi B, Gatti PJ, Haislip AM, Fermin CD, Garry RF (1998) Role of potassium in human immunodeficiency virus production and cytopathic effects. *Virology* 247: 189–199
4. Dunn JP, Jabs DA (1995) Cytomegalovirus retinitis in aids: natural history, diagnosis and treatment. *Aids Clin Rev* 96: 99–129
5. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H et al. (1973) *In vitro* cultivation of human tumours: establishment of cell lines derived from a series of solid tumours. *J Natl Cancer Inst* 5: 1417–1423
6. Glitsch HG (2001) Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiol Rev* 81(4): 1791–1826
7. Hannaert P, Alvarez-Guerra M, Pirot D, Nazaret C, Garay RP (2002) Rat NKCC2/NKCC1 cotransporter selectivity for loop diuretic drugs. *Naunyn Schmiedeberg Arch Pharmacol* 365: 193–199
8. Hartley CE, Buchan A, Randall S, Skinner GR, Osborne M, Tomkins LM (1993) The effects of lithium and potassium on macromolecular synthesis in herpes simplex virus-infected cells. *J Gen Virol* 74: 1519–1525
9. Hendricks RL (1999) Immunopathogenesis of ocular infections. *Chem Immunol* 73: 120–136
10. Jacobs JP, Jones CM, Baille JP (1970) Characteristics of a human diploid cell designated MRC-5. *Nature* 227: 168–170

## Ionic Contra-Viral Therapy for treating DNA virus infections

11. Lauf P (1984) Thiol dependent passive K/Cl transport in sheep red cells: IV. Furosemide inhibition as a function of external  $Rb^+$ ,  $Na^+$  and  $Cl^-$ . *J Membr Biol* 77: 57–62
12. Liesegang TJ (1999) Varicella-zoster eye disease. *Cornea* 18: 511–531
13. Link F, Szanto J, Blaskovic D, Raus J, Dobrocka E, Pristasova S (1966) Interaction of heart glycosides and viruses. *Acta Virol* 10: 455–461
14. Loewe S, Muischnek H (1926) Effect of combinations: a mathematical basis of problem. *Arch Exp Pathol Pharmacol* 114: 313–326
15. Macpherson I, Stoker M (1962) Polyoma transformation of hamster cell clones – an investigation of genetic factors affecting cell competence. *Virology* 16: 147–151
16. Maglova LM, Crowe WE, Russell JM (2004) Perinuclear localisation of Na–K–Cl-cotransporter protein after human cytomegalovirus infection. *Am J Physiol Cell Physiol* 286: C1324–C1334
17. Nagai Y, Maeno K, Inuma M, Yoshida T, Matsumoto T (1972) Inhibition of virus growth by ouabain: effect of ouabain on the growth of HVJ in chick embryo cells. *J Virol* 9: 234–243
18. Nugier F, Colin JN, Aymard M, Langlois M (1992) Occurrence and characterization of acyclovir-resistant herpes simplex virus isolates: report on a two-year sensitivity screening survey. *J Med Virol* 36: 1–12
19. O'Connor T (2000) Assessment of activity of topical virucidal agents. In: Kinchington D, Schinazi RF (eds) *Antiviral methods and protocols*. Humana Press, Totowa, NJ, pp 207–212
20. Patou G, Crow TJ, Taylor GR (1986) The effects of psychotropic drugs on synthesis of DNA and the infectivity of herpes simplex virus. *Biol Psychiatry* 21: 1221–1225
21. Russell JM (2000) Sodium–Potassium–Chloride Cotransport. *Physiol Rev* 80: 211–276
22. Scheiner-Bobis G (2002) The sodium pump; its molecular properties and mechanics of ion transport. *Eur J Biochem* 269: 2424–2433
23. Skinner GRB, Hartley CE, Buchan A, Harper L, Gallimore P (1980) The effect of lithium chloride on the replication of herpes simplex virus. *Med Microbiol Immunol* 168: 139–148
24. Squires KE (2001) An introduction to nucleoside and nucleotide analogues. *Antivir Ther* 6: 1–14
25. Stinski MF (1978) Sequence of protein synthesis in cells infected by human cytomegalovirus; early and late virus-induced polypeptides. *J Virol* 26: 686–701
26. Jeor St S, Hutt R (1997) Cell DNA replication as a function in the synthesis of human cytomegalovirus. *J Gen Virol* 37: 65–73
27. Ulug ET, Garry RF, Bose HR Jr (1996) Inhibition of  $Na^+ K^+$  ATPase activity in membranes of Sindbis virus-infected chick cells. *Virology* 216: 299–308
28. Woodfork KA, Wonderlin WF, Peterson VA, Strobl JS (1995) Inhibition of ATP-sensitive potassium channels causes reversible cell-cycle arrest of human breast cancer cells in tissue culture. *J Cell Physiol* 162: 163–171
29. Ziaie Z, Kefalides NA (1989) Lithium chloride restores host protein synthesis in herpes simplex virus-infected endothelial cells. *Biochem Biophys Res Commun* 160: 1073–1078

Author's address: Christopher Hartley, Henderson Morley Plc, Metropolitan House, 2 Salisbury Road, Moseley, Birmingham B13 8JS, U.K.; e-mail: ch@henderson-morley.com