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Synthesis and characterization of novel dipeptide ester prodrugs of acyclovir

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Abstract

Four dipeptide (Gly–Gly, Gly–Val, Val–Val, Val–Gly) ester prodrugs of 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir, ACV) were synthesized. LC/MS was used to characterize the new prodrugs. Both ^{1}H NMR and ^{13}C NMR spectra of the four prodrugs of ACV were measured and assigned based on spectral comparison with compounds of similar structures.

Keywords: Acyclovir; Dipeptide; Prodrug; Esterfication; ^{1}H NMR; ^{13}C NMR; LC/MS

1. Introduction

A direct synthesis of acyclovir (ACV) (9-[(2-hydroxyethoxy)methyl]guanine) from guanonsine via exchange of glycosyl substituents is the most efficient method reported so far [1,2]. Boryski [3,4] and Boryski and coworkers [5,6] have studied the mechanism of conversion of guanosine to ACV in details. Schaeffer et al. [7] first reported ACV as a highly specific inhibitor of herpes virus proliferation. Because of its poor aqueous solubility and lipophilicity, the intravenous and oral delivery is far from being optimal. The drug cannot be given as aqueous eye drops or by intramuscular injection because of its limited solubility in water. Previously a number of ACV prodrugs were developed with the aim of improving the delivery characteristics. Different approaches have been implemented to serve these aims. Colla et al. [8] and Maudgal et al. [9] have added various amino acid moieties to ACV to form amino acid esters prodrugs, which were potentially useful in the preparation of eye drops formulation. Beauchamp et al. [10] have used similar approach and prepared several amino acid esters prodrugs as potential prodrugs for oral administration. Shao et al. and Yang et al. [11,12] have reported the synthesis, nasal and ocular absorption, and metabolism of

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various aliphatic acid ester prodrugs of ACV. Bundgaard et al. [13] evaluated a series of N-substituted 3- or 4-(amino-methyl) benzoate esters of ACV as solution-stable, biolabile prodrug form. Recently, Gao and Mitra [14] have synthesized several series of N^2-acyl-ACV, α-, β-, γ-amino acid esters and dicarboxylic acid esters of ACV to investigate their effectiveness in enhancing membrane transport.

The amino acid ester prodrugs of ACV have low stability at the physiological pH (7.4) and their delivery is still not optimal. The overall objectives of this study are to synthesize prodrugs of ACV possessing stability, solubility and membrane transport at the physiological pH enabling optimal delivery. The oligopeptide transporter (PEPT1) is an essential mammalian protein that actively transports small peptides (active transport) [15]. PEPT1 is found on the wall of the small intestine of mammals [16,17] and also on the cornea and internal eye structures [18]. Based on these studies, we have chosen the approach of synthesizing dipeptide ester prodrugs of ACV. In addition, dipeptide prodrugs can exist in several more possible conformers than the amino acids prodrugs, which may lead to more stable forms. Also the possibility of intramolecular hydrogen bonding between the carbonyl group of the ester link and the free amino group of the dipeptide to form more stable prodrug, is higher in the case of dipeptide esters than in the case of the amino acid prodrugs. Both geometrical isomerism and hydrogen bonding might enhance the stability of the prodrugs. In some cases increasing the solubility using the prodrug strategy would help in drug delivery by concentration gradient (passive transport) and by transporter mediated delivery (facilitated transport). Such compounds would probably have high potential for oral administration and also eye drops formulation. For the first time, four dipeptide (Gly–Gly, Val–Gly, Gly–Val, Val–Val) ester prodrugs of ACV have been synthesized and characterized to investigate its aqueous solubility, stability, antiviral activity and its membrane delivery. During the synthesis, the intermediate and product structures were confirmed by ^{1}H and ^{13}C NMR, TLC and LC/MS analysis.

2. Materials and techniques

2.1. NMR spectroscopy

The ^{1}H and ^{13}C NMR were recorded on a Bruker AC 250-modified Tecmag DSPect Fourier transform NMR spectrometer at 250 and 63 MHz, respectively, for 0.25–0.5 M DMSO-d_6 solutions at 23 °C. Chemical shifts are expressed in parts per million relative to tetramethylsilane as the internal standard. For the ^{13}C NMR spectra, the spectral width was 20 000 Hz and the number of data points was 16 000, generating a 2.5 Hz per point digital resolution. The flip angle was 4 μs (90°) and the acquisition time was 0.409 s with a pulse delay of 3 s; 500–1000 scans were accumulated for each spectrum.

2.2. Liquid chromatography/mass spectrometry

LC/MS was carried out by coupling an high-performance liquid chromatographic (HPLC) system (Spectra System) to a Finnigan aQa single quadruple mass spectrometer using an electrospray interface held at 350 °C. The electrospray needle was maintained at 3.00 kV with declustering potential set at 30 V. Ultrapure nitrogen was used as nebulizer gas. The mass spectrometer was operated in the positive ion mode. The flow rate was 1.0 ml/min, and a 20-min isocratic of 20% CH_3CN with 0.1 trifluoroacetic acid (TFA) and a C8 column of 10-cm length were used. The compounds were dissolved in CH_3OH (1 mg/ml) and 10 μl was injected directly for the LC/MS analysis.

2.3. Materials

All of the chemicals (Boc–L–Val, Boc–Gly, guanosine, 1,3-dioxolane, *p*-toluenesulfonic acid monohydrate, TFA, dimethylformamide and acidic anhydride) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific Company (St. Louis, MO).

2.3.1. Acyclovir

ACV was synthesized following the method reported by Shiragami et al. [1,2].

2.3.2. Dipeptide ester prodrugs of acyclovir

Boc–Gly–OH (3.11 g, 17.76 mmol) and dicyclohexyl carbodiimide (DCC) (1.83 g, 8.88 mmol) were dissolved in dry DMF (20 ml) under a nitrogen atmosphere. The mixture was continuously stirred for 1 h at 0 °C. A precipitate, *N*,*N*′-dicyclohexylurea, started to separate almost immediately and its amount gradually increased. A solution of ACV (2.00 g, 8.88 mmol) and 4-(*N*,*N*-dimethylamino) pyridine (DMAP) (0.06 g, 0.65 mmol) in DMF (100 ml) was added dropwise to the reaction mixture at 0 °C. The reaction was stirred and allowed to warm up to the room temperature. After 24 h, the reaction was checked by TLC (1:9, methanol to dichloromethane) and LC/MS and it was complete. The urea derivative was removed by filtration and washed with dichloromethane. The solvents of the combined filtrate and washing were partially removed under reduced pressure. The resulting oil was added dropwise to cold ethyl ether and immediately Boc–Gly–ACV was precipitated. The precipitate in the mother liquor was kept in the refrigerator for 5 h. The Boc–Gly–ACV was filtered and washed with small amount of ethyl ether. The solvents of the filtrate were removed under vacuum and again the resulting oil was added dropwise to the cold ethyl ether. This process was repeated three times and the precipitation by the cold ethyl ether afforded a highly pure compound with 92% yield. The product was characterized by LC/MS and NMR.

A solution of Boc–Gly–ACV (3.00 g) in a fresh TFA (60 ml) was stirred at 0 °C for 1 h. The excess acid was removed under vacuum and the oil residue was mixed with toluene (100 ml) and rotary evaporated at 50 °C to provide a white solid material. The product was washed with ethyl ether and dried under reduced pressure to yield Gly–ACV a slightly hygroscopic white powdery material. Elemental analysis indicated that Gly–ACV has 1 mole of TFA.

Gly–ACV (3.11 g) was dissolved in DMF and neutralized by triethylamine (TEA) (2.20 ml). The neutralized Gly–ACV was added to Boc–Gly amino acid anhydride prepared by the same procedure mentioned above. The reaction was initiated at 0 °C and gradually the temperature was raised to room temperature with continuous stirring. The reaction was complete in 5 h. The work-up procedures mentioned earlier were followed. The product Gly–Gly–ACV was highly pure. The same scheme (Fig. 1) was applied to synthesize Val–Gly, Gly–Val and Val–Val of ACV (Fig. 2).

Also we have tried silica gel column chromatography to purify the intermediates and the products. The silica gel optimal separation conditions of all of the compounds were in the range of 1:4–1:9 (methanol to dichloromethane). The products were highly pure ($\approx$98%). The LC/MS and ^{1}H and ^{13}NMR data were listed in Tables 1 and 2 and Table 3, respectively.

3. Results and discussion

3.1. Synthesis

ACV was successfully synthesized following the method reported by Shiragami et al. [1,2]. Synthesis of Gly–Gly–ACV, Val–Gly–ACV, Gly–Val–ACV and Val–Val–ACV dipeptide ester prodrugs of ACV involve (i) formation of N-protected amino acid anhydrides, (ii) coupling of the N-protected amino acid anhydride with ACV, (iii) deprotecting the amino group of the amino acid ester of ACV, (iv) formation of N-protected amino acid anhydride, (v) coupling of the N-protected amino acid anhydride with the amino acid esters of ACV (formation of dipeptide), and (vii) finally deprotection of the amino group of the dipeptide ester of ACV (Fig. 1).

A method to attach ACV, a terminal hydroxy containing model drug to glycine and valine amino acids through ester linkage was developed. Amino acid esters of ACV Gly–ACV and Val–ACV were prepared in three steps (Fig. 1) using a modified procedure of method B reported by Beauchamp et al. [10]. One of the simplest methods for esterification of the hydroxymethyl ACV is to use the symmetrical anhydride of the protected amino acid

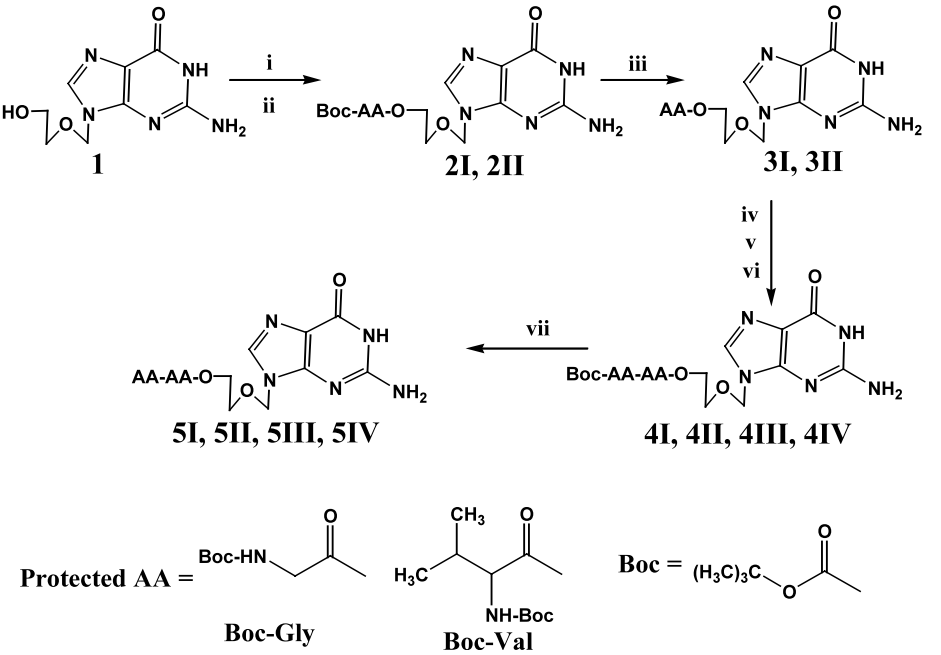

Fig. 1. (i) Boc–AA, DCC, DMF, 0 °C, 1 h, (ii) DMAP, DMF, 24 h, r.t. (iii) TFA, 0 °C, 60 min (iv) Boc–AA, DCC, DMF, 0 °C, 1 h, (v) TEA, r.t., (vi) adding the N-protected amino acid anhydride (step iv) to the neutralized amino acid ester of ACV (step v), 5 h, r.t., (vii) TFA, 0 °C, 60 min.

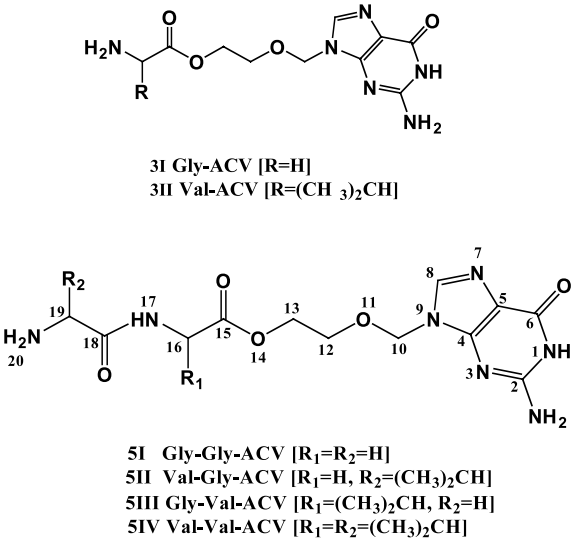

Fig. 2. Structures of Gly–ACV and Val–ACV amino acid esters of ACV and the dipeptide moieties Gly–Gly, Val–Gly, Gly–Val and Val–Val attached to ACV model drug.

in the presence of DMAP. Symmetrical anhydrides are generated under nitrogen atmosphere using two equivalents of Boc-protected amino acid and one equivalent of DCC in DMF. Preformed symmetrical anhydrides have been used by many research groups, mainly in Boc chemistry, because of their excellent reactivity [19,20]. A solution of ACV (1) and DMAP in DMF was added to the reaction mixture. To avoid side products which could be formed as a result of coupling of the amino acid with the amine group of ACV the reaction should be initiated at 0 °C. The choice of amino acid-labile Boc-protecting group was appropriate since no other parallel reactions were observed through the reaction steps.

Boc–AA–ACV compounds were purified by precipitation in cold ethyl ether. The precipitation method afforded highly pure intermediates and products and no hydrolysis of the ester linkage was observed. Beauchamp et al. have reported [10] synthesis of 18 amino acid prodrugs. *N*-methyl-L-alanyl ACV and L-2-aminobutyrate ACV were the only two compounds, which were synthesized by method B using *N*-Boc-protecting group. It is obvious that the real challenge in this method is

Table 1
Yields, measured and calculated m/z ratios of the products

Compound	Yield (%)	Measured MS $[M+1]^+$	Measured MS $[2M+1]^+$	Calculated MS $[M+1]^+$
Gly–Gly–ACV	72	339.7	–	340.13
Val–Gly–ACV	74	381.9	763.3	382.18
Gly–Val–ACV	70	381.9	763.4	382.18
Val–Val–ACV	75	423.9	847.4	424.22

the crystallization of the product from 5% CH_2Cl_2/MeOH. This crystallization condition might result in the hydrolysis of the ester linkage to generate the starting materials. Boc–Gly–ACV also hydrolyzed under similar crystallization conditions. During the LC/MS measurements, it was also observed that Gly–ACV dissolved in methanol, started to hydrolyze within 10 min. After coupling to the Boc-protected glycine and valine amino acids, the protecting group was removed by TFA to give the trifluoroacetate salt of Gly–ACV and L–Val–ACV (Fig. 2). The elemental analysis of Val–ACV and Gly–ACV indicated that each of these prodrugs contains 1 mole of TFA.

Compound (3) (Fig. 1) was neutralized by TEA and treated with N-protected amino acid anhydride for about 5 h. The neutralization step was essential for completing the reaction to form the N-protected ACV dipeptide esters. The resulting products (4) were purified by precipitation following the same procedure in step 2, filtered, dried and the Boc group was deprotected (acidolytic removal) to yield the desired dipeptide ester prodrugs of ACV (5).

3.2. Characterization

Electrospray ionization coupled to a quadruple mass spectrometer is a powerful analytical tool. It enables the intact flight of thermally labile molecules. For each of the four compounds, one unique peak is observed in the LC–ESI/MS total ion

Table 2
^{1}H NMR data of dipeptide ester prodrugs of ACV in DMSO-d_6

^{1}H	ACV	Gly–Gly–ACV	Val–Gly–ACV	Gly–Val–ACV	Val–Val–ACV
1-NH	10.72 bs	10.81 bs	10.89 s	10.83 s	10.89 s
2-NH_2	6.55 bs	6.65 s	6.69 s	6.64 s	6.64 s
8-CH	7.84 s	7.82 s	8.15 bs	8.07 bs	8.14 bs
10-CH_2	5.36 s	5.35 s	5.37 s	5.36 s	5.34 s
12-CH_2	3.47 bs	3.59 s	3.68 bs	3.67 bs	3.68 m
13-CH_2	3.47 bs	4.14 t	4.15 s	4.22 t	4.14 m
OH	4.69 s				
16-CHR_1		3.92 d	4.04 m	4.13 bs	4.14 m
17-NH		–	–	–	–
19-CHR_2		3.65 d	3.89 m	3.67 bs	3.68 m
20-NH_3^+		8.82 bs	8.89 s	8.50 d	8.57 d
$CH(R_1)$				1.95 m	2.01 m
$CH_3(R_1)$				0.83 d	0.88 d
$CH_3(R_1)$				0.83 d	0.88 d
$CH(R_2)$			2.07 m		2.01 m
$CH_3(R_2)$			0.94 d		0.88 d
$CH_3(R_2)$			0.94 d		0.88 d
TFA		7.95 s	7.95 s	7.95 s	7.94 s

Gly–Gly–ACV [$R_1 = R_2 = H$], Val–Gly–ACV [$R_1 = H$, $R_2 = (CH_3)_2CH$], Gly–Val–ACV [$R_1 = (CH_3)_2CH$], $R_2 = H$], Val–Val–ACV [$R_1 = R_2 = (CH_3)_2CH$].

Table 3
^{13}C NMR data of dipeptide ester prodrugs of ACV

^{13}C	ACV	Gly–Gly–ACV	Val–Gly–ACV	Gly–Val–ACV	Val–Val–ACV
2	153.80	154.09	154.02	154.10	154.02
4	151.40	151.45	151.20	151.43	151.00
5	116.37	116.45	115.58	116.24	114.62
6	156.80	156.76	156.45	156.80	156.32
8	137.80	137.65	137.71	137.90	137.60
10	72.00	71.86	72.01	72.08	71.89
12	70.38	66.33	66.27	66.53	66.13
13	59.89	63.60	63.44	63.39	63.13
15		169.43	169.21	171.05	170.95
16		58.60	57.14	57.37	57.57
18		166.79	168.34	166.42	168.21
19		?	?	?	56.89
$CH(R_1)$[a]				30.07	29.75
$CH_3(R_1)$[a]				17.80	17.64
$CH_3(R_1)$[a]				18.77	18.41
$CH(R_2)$[a]			29.66		29.41
$CH_3(R_2)$[a]			17.45		17.92
$CH_3(R_2)$[a]			17.92		17.23

The question mark indicates that signal/noise ratio was too low to identify the signals.
[a] Gly–Gly–ACV [$R_1 = R_2 = H$], Val–Gly–ACV [$R_1 = H$, $R_2 = (CH_3)_2CH$], Gly–Val–ACV [$R_1 = (CH_3)_2CH$], $R_2 = H$], Val–Val–ACV [$R_1 = R_2 = (CH_3)_2CH$].

current and UV chromatograms. The positive electrospray mass spectra of Gly–Gly–ACV, Val–Gly–ACV, Gly–Val–ACV and Val–Val–ACV eluting at approximately 2.45, 2.45, 2.46 and 2.97 min (figures not presented), respectively, give the expected $M+1$ ($M = m/z$) values of these compounds mainly in monomer and dimer forms (Table 1).

The ^1H and ^{13}C NMR spectral assignments of the four compounds are listed in Table 2 and Table 3, respectively. As shown in Table 2 the peak at 4.69 ppm for ACV has been assigned to the proton of the OH group. This peak disappeared upon conversion of OH to O–AA or O–AA–AA (Fig. 1). Also this conversion leaded to deshielded ^1H NMR chemical shifts of 0.67–0.75 ppm for the protons of carbon CH_2OCHO (13-CH_2), to which these groups are attached. Immediate adjacent protons CH_2O (16-CHR_1) also deshielded by $\sim$ 0.4 ppm. These modifications in the ^1H NMR spectra are strong evidence of occurrence of a substitution reaction and formation of the amino acid esters of ACV. The proton on carbon 8 produced some shift towards higher parts per million. In comparison with ACV, other protons of the carbons of the ACV moiety of these dipeptide ester prodrugs of ACV produced almost no changes in the chemical shift either in ^1H NMR or ^{13}C NMR. The assignments of ^{13}C NMR were straightforward. The highest chemical shifts in ^{13}C NMR spectra for each of these compounds were assigned to the carbon COO (15-C) of the carboxyl group and the immediate lower chemical shifts were assigned to the carbon CONH (18-C) of the amide group (Table 3). Both the highest and immediate lower chemical shifts indicated the formation of the ester and peptide bonds, respectively.

Intact cornea and Caco-2 cell permeabilities of all the ACV dipeptide ester prodrugs were observed to be higher than ACV possibly due to recognition of the prodrugs by the PEPT1 on the cornea and Caco-2 cells. Also they exhibited excellent chemical stability and antiviral activity against herpes simplex virus thereby rendering these lead compounds promising drug candidates against herpes virus infections. More details can be found in Refs. [21–24].

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References

[1] K. Shiragami, Y. Koguchi, Y. Tanaka, S. Takamatsu, Y. Uchida, T. Ineyama, K. Izawa, Nucleos. Nucleot. 14 (1995) 337.
[2] K. Izawa, H. Shiragami, Pure Appl. Chem. 70 (1998) 313.
[3] J. Boryski, Nucleos. Nucleot. 15 (1996) 771.
[4] J. Boryski, J. Chem. Soc. Perkin Trans. 2 (1997) 649.
[5] J. Boryski, B. Golankiewiez, Nucleos. Nucleot. 8 (1989) 529.
[6] J. Boryski, A. Manikowski, Nucleos. Nucleot. 18 (1999) 1057.
[7] H.J. Schaeffer, L. Beauchamp, P. de Miranda, G.B. Elion, D.J. Bauer, P. Collins, Nature (Lond.) 272 (1978) 583.
[8] L. Colla, E. De Clercq, R. Busson, H. Vanderhaehe, J. Med. Chem. 26 (1983) 602.
[9] P.C. Maudgal, E. Clercq, J. Descamps, L. Missotten, Arch. Ophthalmol. 102 (1984) 140.
[10] L.M. Beauchamp, G.F. Orr, P. de Miranda, T. Burnette, T.A. Krenitsky, Antiviral Chem. Chemother. 3 (1992) 157.
[11] Z. Shao, G.B. Park, R. Krihnamoorthy, A.K. Mitra, Pharm. Res. 11 (1994) 237.
[12] C. Yang, H. Gao, A.K. Mitra, J. Pharm. Sci. 90 (2001) 615.
[13] H. Bundgaard, E. Jensen, E. Falch, Pharm. Res. 8 (1991) 1087.
[14] H. Gao, A.K. Mitra, Syn. Commun. 31 (2001) 1399.
[15] D. Meredith, C.A.R. Boyd, J. Membrane Biol. 145 (1995) 1.
[16] D.M. Matthews, Protein Absorption: Development and Present State of the Subject, Wiley-Liss, New York, 1991.
[17] V. Ganapathy, M. Brandsch, F.H. Leibach, in: L.R. Johnson (Ed.), Physiology of the Gastro-intestinal Tract, third ed., Raven, New York, 1994, pp. 1773–1794.
[18] B.S. Anand, A.K. Mitra, Pharm. Res. 19 (2002) 1193.
[19] W.R. Sampson, H. Patsiouras, N.J. Ede, J. Peptide Sci. 5 (1999) 403.
[20] T. Johnson, M. Quibell, D. Owen, R.C. Sheppard, J. Chem. Soc. Chem. Commun. 4 (1993) 369.
[21] B.S. Anand, Y.E. Nashed, A.K. Mitra, Curr. Eye Res., in press.
[22] C. Diaz, Y.E. Nashed, H. Alutri, A.K. Mitra, Curr. Eye Res., in press.
[23] B.S. Anand, J. Patel, A.K. Mitra, J. Pharmacol. Exp. Ther. 304 (2003) 731.
[24] B.S. Anand, J.M. Hill, S. Dey, K. Maruyama, P.S. Bhattacharjee, M.E. Myles, Y.E. Nashed, A.K. Mitra, Invest. Ophthal. Vis. Sci., submitted for publication.