Determination of potential solvents for novel N-substituted 5-(phenylamino)uracil derivatives and evaluation of their cytotoxic effects on Vero 76 Cells

Noor Fahitah Abu Hanipah, Noor Farah Omar Ahmad, Minaketan Tripathy, Elena Gureeva, Michail Novikov, Yulia Gushchina, Olga Butranova, Nafeeza Hj Mohd Ismail, Seok Mui Wang, Anna Krasilnikova

Abstract. N-substituted 5-(phenylamino)uracil derivatives have recently shown to possess potential antiviral properties. However, the high lipophilicity of these compounds has limited their ability to be dissolved in aqueous media for further in vitro and in vivo studies. This study aimed to determine the potential solvents for novel N-substituted 5-(phenylamino)uracil compounds and to evaluate the cytotoxic effects of these solvents on Vero 76 cells. Eight solvents, namely acetone, methanol, ethanol, dimethyl sulfoxide (DMSO), polyvinylpyrrolidone, nicotinamide, L-arginine, and sodium benzoate, were used to dissolve 1600 µM each of compound Z214 and compound Z276, which were chosen as the representatives of novel N-substituted 5-(phenylamino)uracil derivatives. Only L-arginine (700 mM), sodium benzoate (1500 mM), and DMSO (128 mM) were able to solubilise both compounds. Cytotoxicity assays on Vero 76 cells have shown that the maximum concentrations of L-arginine, sodium benzoate, and DMSO that demonstrated 100% cell viability were 108 mM, 10 mM, and 211 mM respectively. L-arginine at concentrations ranged from 215 mM to 860 mM have shown to significantly increased cell proliferation; while both sodium benzoate and DMSO have significantly reduced cell viability at concentrations ≥ 10 mM and ≥ 211 mM respectively. CC_{50} values were 23.22 mM and 214.92 mM for sodium benzoate and DMSO respectively. The findings in this study revealed that DMSO at a concentration of 211 mM was found to be the most appropriate solvent to solubilise 1600 µM and below of novel N-Substituted 5-(phenylamino)uracil derivatives.

Keywords: N-substituted 5-(phenylamino)uracil derivatives, cytotoxicity, Vero cells, L-arginine, sodium benzoate, dimethyl sulfoxide
INTRODUCTION

The solubility of the desired chemical compounds is considered as a fundamental process before the evaluation of their effectiveness as a potential drug takes place. The solubility of the compound is a state of the chemical substance called solute dissolved in solid, liquid or gaseous solvent to reach a homogeneous solution. This state is a crucial step as it plays an important role, particularly in testing the activity of the lipophilic compounds in vitro experiment due to the need to dissolve the compounds in hydrophilic media. There are several approaches available to improve the solubility of lipophilic agents. Common organic solvents such as methanol, ethanol, acetone, acetic acid, and dimethyl sulfoxide (DMSO) are widely used in the pharmaceutical industry. The addition of these solvents has shown to enhance the solubility of most of the lipophilic substances (Maes et al., 2012). Apart from that, more recent strategies in increasing the solubility of highly lipophilic substances include the use of mixed solvency concept, the addition of solubilizing enhancers or hydrotropic agents, combination with surfactants, development of solid dispersion, and nanosuspension (Maheshwari & Moondra, 2010; Savjani et al., 2012; Hamzeloo-Moghadam & Taiebi, 2014).

Hydrotropic solubilization technique is one of the potential tools to enhance the solubility of lipophilic substances. Hydrotropic agents are compounds that have amphiphilic molecular structures. The presence of amphiphilic molecular structures has shown to increase the solubility of poorly soluble solutes (Hodgdon & Kaler, 2007; Maheshwari & Moondra, 2010) through the interaction with water-soluble molecules via weak Van der Waals forces (Dhapat & Mehta, 2015). Hydrotropes which can be anionic, cationic or neutral, enhance the aqueous solubility of organic substances by forming aggregation, structure breaker and structure maker, as well as by developing micellar like structure (Dhapat & Mehta, 2015). Recent articles have shown that L-arginine, sodium benzoate, and nicotinamide have been used as hydrotropic agents to enhance drug solubility (Nidhi et al., 2011; Dhapat & Mehta, 2015). Arginine, which is an essential amino acid, serves as a precursor for many important molecules in cell biology. Apart from that, L-arginine has shown to have potential as a solubility enhancement agent (Shukla & Trout, 2010). The mechanism of arginine solubilizing properties is mostly associated with its ability to suppress the aggregation and adsorption of the proteins and other high molecular weight substances to the solid surface (Arakawa et al., 2007; Arakawa et al., 2008; Shukla & Trout, 2010; Shukla & Trout, 2011). Sodium benzoate, which is widely used as a preservative agent in food and pharmaceutical industries, also has shown the ability to solubilise hydrophobic agents (Maheswari et al., 2009; Soni et al., 2014). It is also currently used as a cosolvent in mixed solvency approach to increase the solubility of some lipophilic drugs in aqueous solutions (Soni et al., 2014). Nicotinamide, which is a form of vitamin B3, is found in food and used as a dietary supplement and treatment for pellagra as well as a solubility enhancement agent (Rasool et al., 1991; Agrawal et al., 2004). It is used in the pharmaceutical industry to enhance the solubility of the drugs by forming a stacking complexation with the drug molecules (Kongmuang, 2002; Hussain et al., 1993). The drugs and the complex molecules interact with each other to reduce the exposure of the hydrophobic region to aqueous media (Sanghvi et al., 2007). Nicotinamide also has the ability to break the surface tension and water conductivity to form self-association (Sanghvi et al., 2007). Polyvinylpyrrolidone (PVP), a water-soluble polymer has been shown to serve as a solubilizing agent and function as a binder to the active ingredient to enhance the bioavailability of the drugs. In previous studies, PVP has shown to increase dissolution of the active ingredients and improve bioavailability of poorly water-soluble drugs such as indomethacin (Fini et al., 2008; Srikanth et al., 2010; Barmpalexis et al., 2013).

The non-nucleoside analogues are known to have broad-spectrum of antiviral activity. The chemical structure of most of the non-nucleoside analogues includes the presence of three aromatic rings connected by short linkers (Novikov et al., 2010). The newly synthesized N-substituted 5-(phenylamino)uracil derivatives are non-nucleoside analogues containing aromatic rings with the addition of uracil. The replacement of one aryl ring with uracil potentially offers several advantages such as a great potential of antiviral
activities against RNA viruses as well as advantages in pharmacokinetic properties (Novikov et al., 2010).

Novel uracil contained non-nucleoside analogues have demonstrated positive activity against HIV type 1, Hepatitis C virus (HCV), Epstein-Barr virus (EBV), and Human Cytomegalovirus (HCMV) (Maruyama et al., 2003; Novikov et al., 2010; Novikov et al., 2013). Novel 1-[ω-(phenoxy)alkyl] uracil derivatives (compounds 9–30) were shown to inhibit HCMV with EC$_{50}$ ranging from 8.9 μM to 100 μM (Novikov et al., 2013). The compound 13 from the same group of 1-[ω-(phenoxy)alkyl]uracil derivatives inhibited HIV-1(IIIb) and HIV-2 (ROD) at 24 μM concentration with a 50% cytotoxic concentration of 154 μM (Novikov et al., 2013). In another study, 5-(arylamino)-1-benzyluracil derivatives have been shown to be effective against HCV (JFH1 strain) with reducing function similarities between HCV and dengue virus (DENV). Considering the virus structure and protein functions similarities between HCV and dengue virus, we hypothesized that novel 5-(arylamino)uracil derivatives are also potentially active against DENV and other RNA viruses such as chikungunya virus (CHIKV) and zika virus (ZIKV) (Sun et al., 2005; Mosley et al., 2012; Sofia et al., 2012). However, the poor water-soluble properties of novel N-substituted 5-(phenylamino)uracil derivatives significantly limit their potential to be tested in vitro and in vivo experiments. This study aimed to determine the potential solvents for novel N-substituted 5-(phenylamino)uracil derivatives and to evaluate the cytotoxic effects of these solvents on Vero 76 cells.

**MATERIALS AND METHODS**

**Compounds and solvents**

Novel 1-[3-(phenoxy)benzyl]-5-(phenylamino)uracil and 1-[4-(phenoxy)benzyl]-5-(phenylamino)uracil derivatives were synthesized at the Department of Pharmaceutical and Toxicological Chemistry, Volgograd State Medical University, Russia (Figure 1). Generally both groups of N-substituted 5-(phenylamino)uracil derivatives were prepared by adding a solution containing 6.16 mM of 3-(phenoxy)benzyl or 4-(phenoxy)benzyl bromide in 20 mL of 1,2-dichloroethane to a solution containing 5.98 mM of 2,4-bis(trimethylsilyloxy)-5-(arylamino) pyrimidine in 50 mL of 1,2-dichloroethane. The resulting mixture was refluxed for 14 h, cooled to room temperature, and then added with 10 mL ethanol. The precipitate was filtered and air-dried. The filtrate was evaporated by two thirds and cooled to 0°C. The resulting precipitate was filtered and combined with the previously obtained solid. This was followed by purification using column chromatography and eluting with ethyl acetate/CH2Cl2 (1:1), or by recrystallization from isopropanol/dimethylformamide (2:1). Only 2 compounds, compound Z214 (C25H23N3O3, MW 413.468) and compound Z276 (C27H27N3O3, MW 441.552), were used for solubility test as the representatives of the entire group. Acetone, methanol, ethanol, PVP, nicotinamide, L-arginine, sodium benzoate, and DMSO were chosen as the potential solvents for novel N-substituted 5-(phenylamino)uracil derivatives. All chemicals listed were obtained from Sigma-Aldrich, USA in analytical purity.

**Dissolution of the tested compounds with the potential solvents**

Potential solvents were diluted in serial dilutions in order to find the minimal concentration capable to solubilise 1600 μM of the tested compounds. Dissolution of the tested compounds in different solvents was done under

![Figure 1. General Structure Formula of (a): N-substituted 5-(phenylamino)uracil derivatives containing 4-(phenoxy)benzyl substituent at position 1 of uracil residue; (b): N-substituted 5-(phenylamino)uracil derivatives containing 3-(phenoxy)benzyl substituent at position 1 of uracil residue.](image-url)
standard laboratory conditions and compounds were considered dissolved if the solution remained clear and did not reveal any signs of precipitations or sedimentation for at least 72 h.

**Cell cultures**

Vero (African green monkey kidney) 76 cells were obtained from the American Type Culture Collection (ATCC No. CRL1587). The cells were propagated in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 5% heat-inactivated Fetal Bovine Serum (FBS) (JRS, USA), 1% penicillin (100 units/mL) and 1% streptomycin (100 µg/mL) (HIMEDIA, India), and maintained at 37°C with 5% CO₂ (Rothan et al., 2014). Briefly, Vero 76 cells were cultured in a flask until 80% confluence. The cells were trypsinised, and the number of cells was counted. Vero cells 1×10⁴ in 100 µL DMEM supplemented with 1% of FBS were seeded into each well in 96 well plates and incubated for 24 h. Thereafter, the cells were divided into 4 groups. Group 1 was cultured in 1% DMEM (cell control), and groups 2 to 4 were cultured in 1% DMEM with a selected solvent at various concentration range. The concentrations for the solvents were ranged from 6.75 mM to 860 mM for L-arginine, 0.625 mM to 80 mM for sodium benzoate, and 35.5 mM to 1128 mM for DMSO. The cells were incubated for 72 h at 37°C with 5% CO₂. Morphological observation of treated and control cells was done daily using a fluorescent inverted microscope (IX81, Olympus, Japan). After 72 h incubation, MTS assay was performed to detect cell viability. Each concentration of the tested solvents was done in triplicates and the experiment was repeated 3 times.

**Cell viability assay**

Effects of L-arginine, sodium benzoate, and DMSO on Vero 76 cells viability were estimated using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS) assay (Promega, USA). MTS assay is a modification of tetrazolium reduction assay (MTT), which is a colorimetric assay for assessing cell viability. Tetrazolium assay determines the number of viable cells based on quantitation of metabolic activity in viable cells (Riss & Moravec, 2004). In the presence of NAD(P)H-dependent cellular oxidoreductases, yellow tetrazolium is reduced to purple formazan, the quantity of which is measured by recording the absorbance using a spectrophotometer. Briefly, after 72 h of incubation, 100 µL of media was removed from each well, and 20 µL of MTS was added to each well. The cells were further incubated for 1 h in the dark at 37°C with 5% CO₂. The absorbance was read at 490 nm with the reference wavelength of 630 nm using the plate reader (Tecan Saphire) (Lani et al., 2015). The percentage of cell viability (CV) was calculated using the formula as below:

\[
CV = \frac{\text{Average absorbance of triplicate of drug wells}}{\text{Average absorbance of control wells}} \times 100\%
\]

Percentages of cell viability above 80% were considered as non-cytotoxicity; within 80% - 60% weak; 60% - 40% moderate, and below 40% as a strong cytotoxicity (International Organization for Standardization, 2009). Percentages of cell viability above 110% were considered as cell proliferative effect.

**CC₅₀ determination**

The value of 50% cytotoxic concentration (CC₅₀) on Vero 76 cells for each of the tested solvent was calculated based on cell viability data using dose-response curves plotted in Sigma Plot Software (version 12.0). Firstly, the data were expressed as normalized values of three values of the percentage of cell viability from three independent experiments. After all the values (experimental points) were placed on the graph, the approximation curve was selected. The choice of a particular curve was done based on two criteria: (i) the appearance of the graph (the location of the approximation curve relative to the experimental points); and (ii) the value of the correlation coefficient R that has to be closer to 1. When the approximation curve was selected, the coefficients b and c were determined, and CC₅₀ was calculated based on the equation below:

\[
CC_{50} = -\frac{1}{d} \ln \frac{0.5}{c}
\]

**Statistical analysis**

The experiment was performed in triplicates and repeated at least three times. The results were expressed as the mean ± standard deviation. One-way ANOVA test was performed to compare the
cell viability of each tested concentration against the control. P-value of less than 0.05 was considered as statistically significant.

RESULTS

Solubility of N-substituted 5-(phenylamino)uracil derivatives in different solvents
Several potential solvents were tested to solubilize novel N-substituted 5-(phenylamino)uracil derivatives. Acetone, methanol, ethanol, PVP, and nicotinamide failed to solubilize tested compounds at the concentration of 1600 µM. They did not solubilize 5-(phenylamino)uracil derivatives completely, and the addition of culture medium further resulted in a turbid solution. Whereas, L-arginine, sodium benzoate and DMSO have successfully dissolved 1600 µM of both tested compounds at a minimum concentration of 700 mM, 1500 mM, and 211 mM respectively.

Effect of solvents on Vero cells morphology
Observation of Vero 76 cell morphology in the presence of solvent at 72 h showed that cells exposed to the highest tested concentrations of sodium benzoate and DMSO displayed shrinkage and rounding shape. The cellular detachment was also observed in cells treated with the highest tested concentrations of sodium benzoate and DMSO (Figure 2). Cells treated for 72 h with 860 mM of L-arginine revealed signs of cell proliferation, such as increasing of cell number, cell stacking, and multilayer formation. There were no morphological changes observed on non-treated (cell control) cells or Vero cells exposed to low concentrations of the tested solvents after 72 h of incubation (Figure 2).

Figure 2. Effects of solvents on Vero 76 cell morphology at 72 h post exposure observed by light microscopy (Magnification 10x10). (a), (e), (i): Cell control; (b): L-arginine 6.75 mM; (c): L-arginine 108 mM; (d): L-arginine 860 mM; (f): Sodium benzoate 1.25 mM; (g): Sodium benzoate 10 mM; (h): Sodium benzoate 80 mM; (j): DMSO 35.5 mM; (k): DMSO 211 mM; (l): DMSO 564 mM. The sites of cell shrinkage, rounding shape, and detachment are marked by arrows.
Effects of solvents on cells viability

The cytotoxic effects of L-arginine, sodium benzoate, and DMSO on Vero 76 cells after 72 h of exposure were evaluated using MTS assay. L-arginine did not affect cell viability at concentrations ranged from 6.7 mM to 108 mM, revealing 107.5 ± 2.87% of cell viability (Figure 3). However, at concentrations ranged from 215 mM to 860 mM, a dramatically increased cell proliferation up to 180% was observed (Figure 3). Sodium benzoate at concentrations from 0.625 mM to 10 mM was able to maintain cell viability close to 100%. On the contrary, the exposure of the concentration above 20 mM resulted in a significant dose-dependent reduction in cell viability (Figure 4). DMSO showed no cytotoxic effect at concentrations ranged from 35.5 mM to 211 mM, maintaining the cell viability from 98.11 ± 3.66% till 106.40 ± 2.87% (Figure 5). Nonetheless, an increasing concentration of DMSO more than 211 mM was associated with a significant reduction of cell viability (Figure 5).

Figure 3. Effect of L-arginine on Vero 76 cell viability after 72 h incubation. Each bar represents the mean of percentage of viable cells ± SE. *p-value < 0.05 vs cell control.

Figure 4. Effect of sodium benzoate on Vero 76 cell viability after 72 h incubation. Each bar represents the mean of percentage of viable cells ± SE. *p-value < 0.05 vs control, ***p-value < 0.001 vs cell control.
**Figure 5.** Effect of DMSO on Vero 76 cell viability after 72 h incubation. Each bar represents the mean of percentage of viable cells ± SE. ***p-value < 0.001 vs cell control.

**CC₅₀ determination**

CC₅₀ for sodium benzoate and DMSO were calculated by nonlinear regression (Figure 6, Figure 7). CC₅₀ values for sodium benzoate and DMSO were determined as 23.22 mM and 214.92 mM respectively, indicating sodium benzoate is more cytotoxic to Vero 76 cells compared to DMSO (Table 1).

**Table 1.** CC₅₀ of L-arginine, Sodium benzoate and DMSO on Vero 76 Cells after 72 h of exposure.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>R</th>
<th>CC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine</td>
<td>-</td>
<td>&gt; 860</td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>0.97</td>
<td>23.2233</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.95</td>
<td>214.9229</td>
</tr>
</tbody>
</table>

R - correlation coefficient

**Figure 6.** Determination of CC₅₀ of sodium benzoate on Vero 76 cells using SigmaPlot Software. The data are expressed as normalized values of three independent experiments. CC₅₀ of sodium benzoate is 23.22 mM.

N-substituted 5-(phenylamino)uracil solvents and cytotoxic effects

**DISCUSSION**

The newly synthesized N-substituted 5-(phenylamino)uracil derivatives are non-nucleoside uracil analogues with high potential to be developed as antiviral drugs against RNA viruses (Novikov et al., 2010). Being highly lipophilic substances, these compounds have some limitations for further *in vitro* studies as the cells are maintained in aqueous media. Vero 76 cells are a continuous cell lineage, and it is one of the most common mammalian cell lines used for virology research. Vero cells do not produce interferon and, therefore, this cell line is one of the best *in vitro* models for testing of new antiviral drugs (Emeny & Morgan, 1979; Matskevich et al., 2009). Vero 76 cells are also used to produce both live and inactivated poliovirus, rotavirus, smallpox, etc. vaccines, as well as for propagation of different viruses including Rabies virus, Reovirus, and Japanese encephalitis virus (Barrett et al., 2009; Murray et al., 2017). Apart from virology studies, Vero 76 cells are widely used for propagation of intracellular bacteria and parasites (Singh et al., 2013). Thus, testing the cytotoxic effect of different solvents on Vero cells provides useful data not only in virology but also benefits bacteriology and parasitology.

In this study, three solvents namely L-arginine, sodium benzoate and DMSO, were able to dissolve 1600 µM of Z214 and Z276 compounds to produce a stock concentration for further *in vitro* studies on Vero 76 cells. Based on the cytotoxic activity of 5-(arylamino)-1-benzyluracil derivatives on CEM SS cells, which included compound 11 with CC_{50} of 168 µM, compounds 2, 3, 8, 12, 13 (CC_{50} > 100 µM) and compounds 4, 5 (CC_{50} > 200 µM) (Novikov et al., 2010), the stock concentration of 1600 µM for both Z214 and Z276 were chosen as it allowed further testing of cytotoxicity and antiviral effects at concentration range up to 800 µM. Since these solvents were able to solubilize the tested compounds, the determination of their toxicity on Vero 76 cells is considered important as ideally, a solvent should not cause any undesired effect to the tested cells. The determination of the cytotoxicity of these solvents on Vero 76 cells was performed at 72 h of exposure. Considering novel N-substituted 5-(phenylamino)uracil derivatives to be screened against RNA viruses and primarily against DENV, the choice of 72 h was based on the peak post-infection titers of DENV in Vero cells as described in Lang et al., 2016. The

![Figure 7. Determination of Cytotoxicity of DMSO on Vero 76 cells using SigmaPlot Software. The data are expressed as normalized values of three independent experiments. CC_{50} of DMSO is 214.92 mM.](image-url)
cytopathic effect of ZIKV in C6/36 and Vero cell cultures was also found to be more evident at 72 h post-infection (Barreto-Vieira et al., 2017).

L-arginine is known to have low toxicity for cell culture. In our current study, L-arginine did not reveal any cytotoxic effects at the tested concentrations. However, it appeared to enhance the growth of Vero 76 cells at concentrations from 215 to 860 mM that correlates with the previously reported study, which showed to have the similar proliferative effect of L-arginine at 200 μM on human endometrial RL-95-2 cells (Greene et al., 2013). L-arginine also stimulated proliferation and prevented the lipopolysaccharide-induced death of intestinal cells (Tan et al., 2010). The presence of L-arginine-induced proliferative effect can complicate the assessment of antiviral activity of potential antiviral compounds and makes L-arginine an unsuitable solvent for future testing of in vitro antiviral activity on Vero 76 cells. On the other hand, the ability of L-arginine to stimulate cell proliferation could suggest the potential role of L-arginine as a wound-healing agent as discussed in a review by Alexander and Supp, 2014 (Alexander & Supp, 2014). In other studies, L-arginine also has shown to have anti-aging effect associated with some advance in the immune response and reduced risk of cardiovascular diseases (Gokce, 2004; Gad, 2010).

Sodium benzoate at the concentration of 20 mM was shown to produce a weak cytotoxic effect (73 ± 7.49% of cell viability), that was corresponding to the concentration of 2 mg/ml that had been found to increase chromosomal break and mutagenic effect in lymphocytes (Pongsavee, 2015). However, the cells demonstrated a dramatic decline in viability at doses of more than 20 mM. Due to the relatively high cytotoxicity on Vero 76 cells, particularly at higher concentrations, sodium benzoate was also excluded as an appropriate solvent for the novel N-substituted 5-(phenylamino)uracil derivatives.

DMSO is one of the common solvents used in the pharmaceutical industry. DMSO is characterised by low viscosity and has a high solubilizing capacity for polar and non-polar compounds (Da Violante et al., 2002; Galvao et al., 2014). It is commonly used to solubilize hydrophobic drugs in cell culture studies. However, the data regarding DMSO cell culture toxicity is quite controversial and dependent on the type of cells. Galvao et al. (2014) reported DMSO did not produce a toxic effect on retinal ganglion cells at the concentrations of less than 10% (Galvao et al., 2014). The study on Caco2/TC7 cells also has shown that DMSO was non-toxic at the concentrations up to 10% (Da Violante et al., 2002). While other studies have shown that only the concentrations of 1% or lower are non-toxic (Sharma et al., 2014). In our current study, we also have found that DMSO was able to maintain cell viability on Vero 76 cells at the concentration of 211 mM which corresponds to 1.5% solution.

We accept that these results are limited for Vero 76 cells only and for completeness of the study, it is necessary to test the cytotoxic effect of these solvents on other cells lines commonly used in virology, such as Huh-7, BHK cells, and HEK-293 cells.

CONCLUSION

Based on the results of this study, we conclude that DMSO at the concentration of 211 mM (1.5%) is the most suitable agent to solubilize N-substituted 5-(phenylamino)uracil derivatives for further screening of potential antiviral activity against RNA viruses on Vero cells in vitro.

ACKNOWLEDGEMENTS

This work was supported in part by Universiti Teknologi MARA internal grants: Grant No.: 600-IRMI/DANA5/3/LESTARI (0087/2016) and Grant No.: 600-IRMI/DANA5/3/ BESTARI (00015/2016).

REFERENCES


