In Vitro Cellular & Developmental Biology - Animal (2020) 56:34–41
https://doi.org/10.1007/s11626-019-00418-4

New-Generation Benzimidazole-Based Plasmid Delivery Reagents with High Transfection Efficiencies on the Mammalian Cells

Furkan Ayaz[1] · Ronak Haj Ersan[2] · Burak Kuzu[2,3] · Oztekin Algul[2]

Received: 14 May 2019 / Accepted: 31 October 2019 / Published online: 2 January 2020 / Editor: Tetsuji Okamoto

Abstract

Gene transfer and gene therapy studies require high-efficiency gene delivery reagents. By transferring the piece of DNA that we are interested in, we can alter the expression of certain gene or genes to further characterize its role in the cell function or in the organism's development, metabolism, immune system, etc. Transfection reagents that enable efficient delivery of the DNA to the cells are important tools in the molecular and cellular biology studies. There are chemical products and tools that have been used for transfection of the cells but they are not as efficient as desired or they can induce cytotoxicity. It is crucial to design and generate new transfection reagents to further support the field of biotechnology, molecular studies, cellular biology, and *in vitro* studies relying on them. The more efficient and the less cytotoxic compounds will be especially useful for the field. We synthesized a new set of benzimidazole-based transfection reagents that have higher efficiency to carry GFP expressing plasmid in to the mammalian cells compared with the commercially available ones with low cytotoxicity. GFP expression levels were tracked by flow cytometry to determine the transfection efficiencies. Benzimidazole-based transfection reagents can be safely used for transfection studies in tissue culture as well as in gene therapy applications due to their high efficiency in the gene transfer to the mammalian cells.

Keywords Substituted benzimidazole · Transfection · Cell culture · Tissue culture · Gene Therapy

Introduction

Gene delivery into the mammalian cells is a crucial biotechnological tool in order to study the function of a particular gene and its protein product. By overexpressing or silencing a certain gene or gene groups, we can study that particular gene or gene group products' intracellular function in comparison with unmanipulated cells (Pautz et al. 1993, Holmen et al. 1995, Martinou et al. 1995, Hamilton and Baulcombe 1999, Vacik et al. 1999, Elbashir et al. 2001, Dass et al. 2002, Pampinella et al. 2002, Brazas and Hagstrom 2005, Grunweller and Hartmann, 2005, Recillas-Targa, 2006, Cuerrier et al. 2007, Yamamoto et al. 2009, Kim and Eberwine 2010).

The gene of interest is usually carried on a plasmid that has the machinery for its expression in the mammalian cellular system. These plasmids are expression vectors that can enable either the overexpression of a gene or its silencing by expressing shRNAs that would target the gene of interest's mRNA and lead to its degradation (Pautz et al. 1993, Holmen et al. 1995, Martinou et al. 1995, Hamilton and Baulcombe 1999, Vacik et al. 1999, Elbashir et al. 2001, Dass et al. 2002, Pampinella et al. 2002, Brazas and Hagstrom 2005, Grunweller and Hartmann, 2005, Recillas-Targa, 2006, Cuerrier et al. 2007, Yamamoto et al. 2009, Kim and Eberwine 2010). Plasmid cannot go through the cellular membrane by itself and it requires a transfection reagent during its journey into the cell.

There have been commercially available transfection reagents (Gao and Huang 1991, Harpe et al. 2000, Simberg et al. 2000, Kiefer et al. 2004, Geisse and Henke 2005, Ehrhardt et al. 2006, McBain et al. 2007, Hunt et al. 2010, Parelkar et al. 2011, Park et al. 2012, Melissa et al. 2013). They have been improved but the field still needs better and economical transfection reagents that would enable the

✉ Furkan Ayaz
furkanayaz@mersin.edu.tr

✉ Oztekin Algul
oztekinalgul@mersin.edu.tr

[1] Department of Biotechnology, Faculty of Arts and Science Mersin University, 33110 Mersin, Turkey

[2] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mersin University, 33169 Mersin, Turkey

[3] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Van Yuzuncu Yil University, Van, Turkey

highest transfection efficiency with the least cytotoxic effect (Pautz et al. 1993, Holmen et al. 1995, Martinou et al. 1995, Hamilton and Baulcombe 1999, Vacik et al. 1999, Ghosh et al. 2000, Elbashir et al. 2001, McGregor et al. 2001, Dass et al. 2002, Pampinella et al. 2002, Brazas and Hagstrom 2005, Grunweller and Hartmann, 2005, Recillas-Targa, 2006, Cuerrier et al. 2007, Yamamoto et al. 2009, Kim and Eberwine 2010, Parelkar et al. 2011, Brazas and Kirschner et al. 2006). Gene delivery reagents are taking more attention due to the gene therapy studies and high demand for efficient and biocompatible transfection tools and techniques in cellular and molecular biology applications (Holmen et al. 1995, Carter and Samulski 2000, Ghosh et al. 2000, Kircheis et al. 2001, McGregor et al. 2001, Brazas and Kirschner et al. 2006, Seow and Wood 2009, Slowing et al. 2010, Parelkar et al. 2011, Nimesh et al. 2015). In this study, we aimed to synthesize benzimidazole-based transfection reagents and further characterize their transfection efficiencies as well as cytotoxicities in comparison with commercially available Xtremegene HP product (Liue et al. 2017). GFP expressing mammalian expression plasmid was used for the trials on the mammalian macrophages RAW 264.7 cells (Moyano et al. 2016, Ayaz 2018, Ayaz 2018, Ayaz et al. 2019). These cells were chosen for our studies since these are one of the most common and well-characterized cell types used in *in vitro* immunology studies (Moyano et al. 2016, Ayaz 2018, Ayaz 2018, Ayaz et al. 2019).

Based on our results, we generated benzimidazole-based transfection reagents with a couple of fold higher transfection efficiencies compared with the Xtremegene HP. Moreover, these products had comparably cytotoxic effects as Xtremegene HP. These derivatives can be useful in transfection studies and after further characterization in the gene therapy applications.

Materials and Methods

1. Synthesis of ORT series of benzimidazole derivatives:

2. Preparation of ORT series of benzimidazole derivatives:

Substituted benzimidazole derivatives of this study were synthesized by using Phillip's method (Nannapaneni et al. 2010). Equimolar concentrations of the substituted diamine groups were refluxed with the corresponding carboxylic acids in 5 N hydrochloric acid for 5–20 h. The mixture was cooled down in ice bath and it was neutralized with ammonia. The resulting precipitates were filtered off to get rid of the solvent. The precipitates were washed three times with water and purified by recrystallization.

3. *In vitro* cell culture studies:

Cell growth for the macrophage cell line. RAW 264.7 macrophage cells were obtained from ATCC on 15 December 2017 and incubated in the Roswell Park Memorial Institute media (RPMI 1640), 10% fetal bovine serum, 1% antibiotic (100 μg/mL penicillin and 100 μg/mL streptomycin), and sodium pyruvate containing media at 37°C 5% CO_2 incubator. The cells did not have mycoplasma when the InvivoGen mycoplasma test kit was used on them on 15 December 2017, and the cells were authenticated by stimulating them with LPS which lead to substantial amounts of pro-inflammatory cytokine TNFα secretion on 25 December 2017. The cells were passaged into new flasks in fresh complete RPMI as described above, after they reached confluency once in every 4 d. The cells were used at third passage.

Mammalian cells' transfection protocol. Roche Xtremegene HP protocol was strictly followed with 3:1 ratio of reagent to plasmid based on our previous studies and the transfection reagents protocol; this was determined as the optimal working ratio. A total of 10^6 RAW 264.7 cells in 1 mL were seeded in 24-well plates and rested overnight at 37°C 5% CO_2 incubator. ORT series of molecules were soluble in ethanol as Xtremegene HP. These derivatives were dissolved in ethanol and after a cytotoxicity test; 12 μg/mL final concentration of those compounds was used for transfection studies. pOPINEneo-3C-GFP plasmid (pOPINEneo-3C-GFP was a gift from Ray Owens (Addgene plasmid # 53534 ; http://n2t.net/addgene:53534 ; RRID:Addgene_53534)) was used for the transfection studies. The plasmid was propagated through colonies of *Escherichia coli* that were grown in LB. The plasmid was later isolated from the bacteria by strictly following the protocol of GeneJET plasmid miniprep kit (K0502 Thermo Scientific EU Lithuania). This plasmid expresses GFP in mammalian cells; therefore, it would enable convenient tracking of the transfected cells by GFP expression. Moreover, the plasmid had a Neomycin resistance gene as a mammalian selection marker. After 48 h of transfection, 400 μg/mL of Neomycin was added into 1 mL of each transfected group's media for selecting only the plasmid expressing efficiently transfected cells. The selection process continued for 96 h before the flow cytometry analysis for GFP expression levels. Xtremegene HP was used as positive control. These experiments were repeated at least in 3 biologically independent experimental set up to reach statistical significance.

FITC/GFP expression analysis of transfected cells by flow cytometry. As described above, after 96 h of Neomycin selection, the cell media was gently aspired from each well and the cells were washed 3 times with 1 mL 1% FBS

PBS. Afterwards, the cells were resuspended in 1% FBS PBS and further analyzed by flow cytometry for the FITC (GFP) expression levels and percentages of GFP expressing cells.

Determination of cytotoxicity levels by trypan blue staining. After discarding the supernatants of the cells that were selected by Neomycin for 96 h, the remaining cells were counted by trypan blue staining to determine the live versus dead cell percentages.

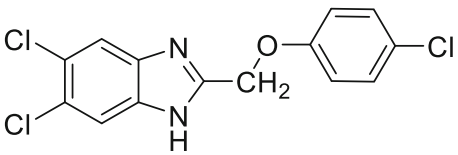
4. Statistical Analysis

The GraphPad Prism Software version 5 was utilized for the statistical analysis. Two paired Student's *t* test was conducted on each data set, $p < 0.0001$, $N = 3$.

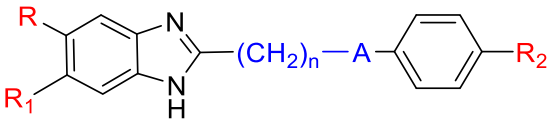
Results

Chemistry. The synthesis routes of the benzimidazole derivatives (ORT-59, ORT-61, ORT-87, ORT-88, ORT-89, ORT-93, ORT-95, ORT-96, and ORT-97) are illustrated in Fig. 1. By using Philip's method, the commercially available substituted 1,2-phenylendiamine derivatives were treated with a suitable carboxylic acid in the presence of HCl to produce the desired benzimidazole derivative. Structures of the compounds are shown in Fig. 2.

5,6-Dichloro-2-((4-chlorophenoxy)methyl)-1H-benzo[d]imidazole (ORT-59)



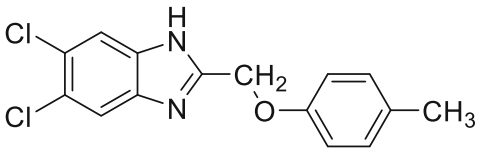
The above procedure was followed with 4,5-dichlorobenzene-1,2-diamine (5348-42-5, ABCR, Karlsruhe, Gennany) and 2-(4-chlorophenoxy)acetic acid (122-88-3, ABCR) to yield ORT-59 as a dark brown crystalline solid (55% yield). The crystallization solvent was ethanol. Rf (Hexanes: EtOAc 50:50) = 0.64; mp = 190°C; IR (KBr, cm^{-1}) V_{max} 3030, 2971, 1445,1231, 1049, 819; ^{1}H NMR (400 MHz, DMSO-d6) δ 11.04 (s, 1H, NH), 7.88 (s, 2H, Ar-H), 7.46-7.32 (m, 2H, Ar-H), 7.21–7.04 (m, 2H, Ar-H), 5.38 (d, J = 13.96 Hz, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d6) δ 136.7, 152.7, 129.3, 125.2, 116.7, 63.9.



R: R1: R2= H, Cl, F, CH_3 **n**= 1, 2 **A**= non, O

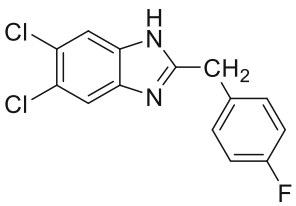
Figure 2. Structure of the derivatives: ORT-96 was designed and synthesized for the first time by our group. ORT-59, ORT-61, ORT-87, ORT-93, and ORT-97 are all commercially available compounds. ORT-88, ORT-89, and ORT-95 compounds were previously synthesized by other research groups but were tested for their gene delivery activity for the first time by our group (Santos et al. 2013, Mentese et al. 2013, Wang et al. 2006). Finally, all compounds (ORT-59, ORT-61, ORT-87, ORT-88, ORT-89, ORT-93, ORT-95, ORT-96, and ORT-97) were synthesized in our lab by our group.

5,6-Dichloro-2-((p-tolyloxy)methyl)-1H-benzo[d]imidazole (ORT-61)



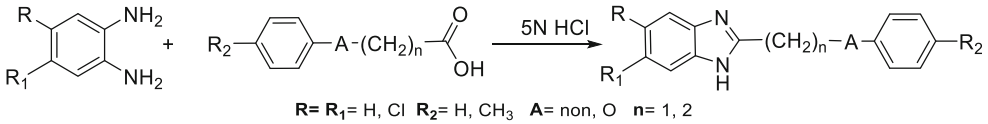
The above procedure was followed with 4,5-dichlorobenzene-1,2-diamine (5348-42-5, ABCR) and 2-(p-tolyloxy)acetic acid (940-64-7, ABCR) to yield ORT-61 as a shiny purple sheets (55% yield). The crystallization solvent was ethanol-water. Rf ($CHCl_3$) = 0.45; mp = 170°C; IR (KBr, cm^{-1}) V_{max} 3061, 3031, 2919, 1507, 1225, 808; ^{1}H NMR (400 MHz, DMSO-d6) δ 12.98 (s, 1H, NH), 7.84 (s, 2H, Ar-H), 7.21–6.94 (m, 4H, Ar-H), 5.31 (s, 2H, CH_2), 2.25 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d6) δ 155.7, 155.2, 130.1, 129.9, 124.4, 14.7, 63.7, 20.0.

5,6-Dichloro-2-(4-fluorobenzyl)-1H-benzo[d]imidazole (ORT-87)



The above procedure was followed with 4,5-dichlorobenzene-1,2-diamine (5348-42-5, ABCR) and 2-(4-fluorophenyl)acetic acid (405-50-5, ABCR) to yield

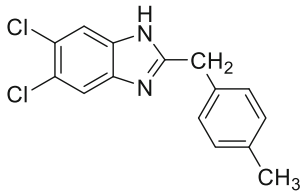
Figure 1. Synthesis of benzimidazole derivatives.



R= R1= H, Cl **R2**= H, CH_3 **A**= non, O **n**= 1, 2

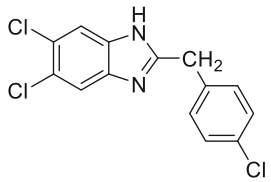
ORT-87 as a shiny light purple powder (63% yield). The crystallization solvent was ethanol-water. Rf ($CHCl_3$: MeOH 95:05) = 0.52; mp = 201°C; IR (KBr, cm^{-1}) V_{max} 3054, 2941, 2732, 1509, 1231, 854; ^1H NMR (400 MHz, DMSO-d6) δ 7.92(s, 2H, Ar-H), 7.44 (s, 2H, Ar-H), 7.22 (d, J = 8.91 Hz, 2H, Ar-H), 4.33 (s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d6) δ 156.1, 131.0, 115.9, 115.8, 115.6, 115.5, 115.4, 115.3, 33.0.

5,6-Dichloro-2-(4-methylbenzyl)-1H-benzo[d]imidazole (ORT-88) (Mentese et al. 2013)



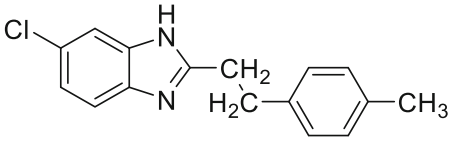
The above procedure was followed with 4,5-dichlorobenzene-1,2-diamine (5348-42-5, ABCR) and 2-(p-tolyl)acetic acid (622-47-9, ABCR) to yield ORT-88 as a light purple powder (69% yield). The crystallization solvent was ethanol-water. Rf ($CHCl_3$) = 0.33; mp = 184°C; IR (KBr, cm^{-1}) V_{max} 3082, 3002, 2845, 1514, 1444, 1288, 865; ^1H NMR (400 MHz, DMSO-d6) δ 12.55 (s, 1H, NH); 7.70 (s, 2H, Ar-H); 7.10–7.19 (m, 4H, Ar-H); 4.11 (s, 2H, -CH2); 2.23 (s, 3H,-CH3).

5,6-Dichloro-2-(4-chlorobenzyl)-1H-benzo[d]imidazole (ORT-89) (Mentese et al. 2013)



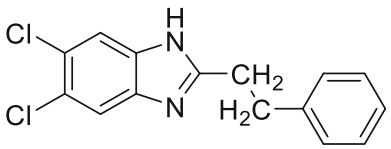
The above procedure was followed with 4,5-dichlorobenzene-1,2-diamine (5348-42-5, ABCR) and 2-(4-chlorophenyl)acetic acid (1878-66-6, ABCR) to yield ORT-89 as a beige powder (71% yield). The crystallization solvent was ethanol-water. Rf ($CHCl_3$: MeOH 95:05) = 0.47; mp = 216°C; IR (KBr, cm^{-1}) V_{max} 3097, 3002, 2843, 1489, 1288, 801; ^1H NMR (400 MHz, , DMSO-d6) δ 12.60 (s, 1H, NH); 7.65–7.77 (m, 2H, Ar-H); 7.29–7.39 (m, 4H, Ar-H); 4.16 (s, 2H, -CH_2).

6-Chloro-2-(4-methylphenethyl)-1H-benzo[d]imidazole (ORT-93)



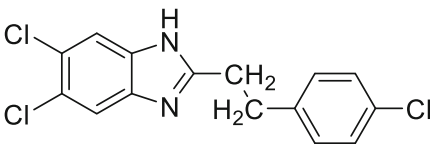
The above procedure was followed with 4-chlorobenzene-1,2-diamine (95-83-0, ABCR) and 3-(p-tolyl)propanoic acid (1505-50-6, ABCR) to yield ORT-93 as a light orange crystalline solid (62% yield). The crystallization solvent was ethanol-water. Rf ($CHCl_3$) = 0.37; mp = 150°C; IR (KBr, cm^{-1}) V_{max} 3171, 3013, 2923, 1512, 1290, 805; ^1H NMR (400 MHz, DMSO-d6) δ 12.43 (s, 1H, NH); 7.81–7.41 (m, 2H, Ar-H), 7.19–7.04 (m, 5H, Ar-H), 3.09 (d, J = 5.17 Hz, 4H, CH_2), 2.26 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d6) δ 137.7, 134.9, 128.9, 121.3, 32.8, 30.4, 20.6.

5,6-Dichloro-2-phenethyl-1H-benzo[d]imidazole (ORT-95) (Wang et al. 2006)



The above procedure was followed with 4,5-dichlorobenzene-1,2-diamine (5348-42-5, ABCR) and 3-phenylpropanoic acid (501-52-0, ABCR) to yield ORT-95 as a brown powder (23% yield). The crystallization solvent was ethanol-water. Rf ($CHCl_3$) = 0.28; mp = 260°C; IR (KBr, cm^{-1}) V_{max} 3057, 3023, 2952, 2711, 1568, 800, 699; ^1H NMR (400 MHz, DMSO-d6) δ 8.00 (s, 2H, Ar-H); 7.33–7.20 (m, 5H, Ar-H), 3.34 (dd, J = 7.15 Hz, J = 8.89 Hz, 2H,CH_2), 3.20 (t, J = 7.81 Hz, 2H, CH_2).

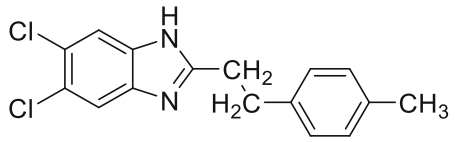
5,6-Dichloro-2-((4-chlorophenyl)ethyl)-1*H*-benzimidazole (ORT-96)



The above procedure was followed with 4,5-dichlorobenzene-1,2-diamine (5348-42-5, ABCR) and 3-(4-chlorophenyl)propanoic acid (2019-34-3, ABCR) to yield ORT-96 as a dark-brown crystalline solid (61% yield). The crystallization solvent was ethanol. Rf (Hexanes/EtOAc 50:50) = 0.63; mp = 223–225°C; IR (KBr, cm^{-1}) V_{max} 3076, 2605, 1406, 1098, 822; ^1H NMR (400 MHz, $(CD_3)_2SO$) δ 7.77 (s, 2H), 7.35 (d, J = 8.56 Hz, 2H), 7.28 (d, J = 8.56 Hz, 2H), 3.13 (s, 4H); ^{13}C NMR (100 MHz,

$(CD_3)_2SO$) δ 157.1, 139.7, 130.7, 130.1, 128.2, 123.6, 32.2, 30.0; LC-MS/MS (ESI+): m/z = 325.1 ($[M+1]^+$).

5,6-Dichloro-2-(4-methylbenzyl)-1H-benzo[d]imidazole (ORT-97)



The above procedure was followed with 4,5-dichlorobenzene-1,2-diamine (5348-42-5, ABCR) and 3-(p-tolyl)propanoic acid (1505-50-6, ABCR) to yield ORT-97 as a brown crystalline solid (59% yield). The crystallization solvent was ethanol-water. Rf ($CHCl_3$) = 0.27; mp = 193°C; IR (KBr, cm^{-1}) V_{max} 3083, 3002, 2914, 2845, 1444,1396, 865; ^1H NMR (400 MHz, DMSO-d6) δ 12.56 (s, 1H, NH), 7.74 (s, 2H, Ar-H), 7.15–6.96 (m, 4H, Ar-H), 3.35 (s, 2H, CH_2), 2.51 (s, 2H, CH_2), 2.24 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d6) δ 157.4, 138.0, 135.0, 128.9, 128.0, 123.5, 32.6, 30.4, 20.6.

Benzimidazole derivatives and Xtremegene HP had comparable low cytotoxic effects on the mammalian macrophages.

Trypan blue staining was done to measure the cytotoxicity of our reagents. GFP expressing plasmid and 6 μL (12 μg/mL) of ORT molecule mixtures were applied to the macrophages for 48 h in a 37°C 5% CO_2 incubator. Positive control groups were treated with plasmid DNA and 6 μL of Xtremegene HP mixture (the same volume as ORT molecules). Negative control wells were treated with 6 μL of ethanol (the solvent) and plasmid DNA mixture. We did not change the media of the cells after transfection, and 48 h past the transfection, the cells were counted in trypan blue. ORT molecules had comparable effects as that of Xtremegene HP (Fig. 3). There was almost 20% decrease in the cells viability in the presence of our molecules, but in some cases, this decrease was as high as 30 to 35% (Fig. 3). In terms of cytotoxicity, ORT 59–89 would be the best candidates to carry on the transfection studies but transfection efficiencies are also a determining factor to choose the best candidate for the application.

Benzimidazole-based compounds had a couple of fold higher transfection efficiencies compared with the Xtremegene HP. In order to prevent any bias against the commercial product that we are comparing with, we strictly followed their protocol. A 3:1 volume ratio of the reagent to plasmid was used to transfect the macrophages. A total of 10^6 RAW 264.7 cells were seeded in 1 mL into 24-well plates and rested overnight in a 37°C 5% CO_2 incubator. Our compounds were dissolved in ethanol as Xtremegene HP. A total of 6 μL of ORT molecules (12 μg/mL) were used in 1 mL of final volume for the transfection. The pOPINEneo-3C-GFP plasmid expresses GFP in mammalian cells and it was targeted to the cells. Forty-eight hours post transfection, the selection of transfected cells started by adding 400 μg/mL of Neomycin into 1 mL of each transfected group's media. The transfected cells were selected for 96 h; this process also pushes the cells to express the GFP while resisting against Neomycin. In positive control wells, the plasmid and Xtremegene HP mixture was applied. In the negative control groups, the plasmid was mixed with 6 μL of the ethanol. After 96 h, the cells were analyzed by flow cytometry for the FITC (GFP) expressing cell percentages. Transfection efficiencies are correlated with the transfected cell percentages. Compared with the Xtremegene HP positive control group, ORT 96 leads to 3.5-fold increase in the GFP positive cell percentages while ORT 59 was 2.5-fold more efficient than Xtremegene HP (Fig. 4). ORT 88, 93, 95, and 97 had almost 1.5-fold higher efficiency compared with Xtremegene HP (Fig. 4). These differences were statistically significant, and therefore, ORT series of benzimidazole-based transfection reagents can be a good alternative to Xtremegene HP.

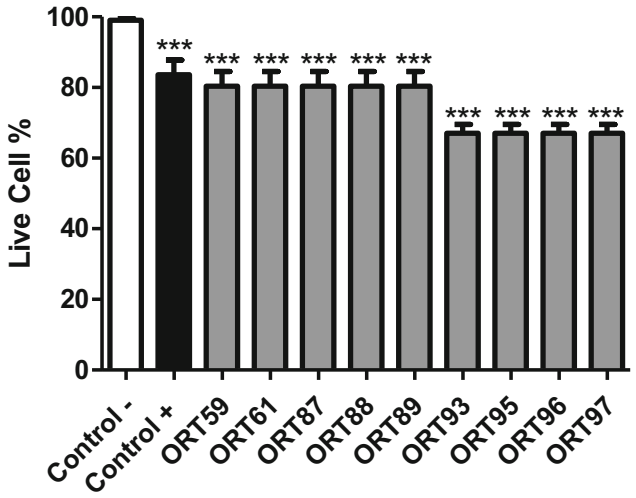


Figure 3. Trypan blue staining was done to determine the live cell percentages of the RAW 264.7 macrophage cells after 48 h of incubation with 12 μg/mL of ORT series of molecules and GFP expressing plasmid mixture at a 37°C 5% CO_2 incubator. A total of 6 μL of ethanol and plasmid mixture was put into the negative control wells. A total of 6 μL of Xtremegene HP together with plasmid was put on the positive control cells. These experiments were conducted in triplicates, $p < 0.001$ and $N = 3$.

Discussion

Gene delivery studies are conducted not only to determine the function of a gene by either increasing or decreasing its expression levels but also for gene therapy purposes (Pautz et al. 1993, Holmen et al. 1995, Martinou et al. 1995, Hamilton and

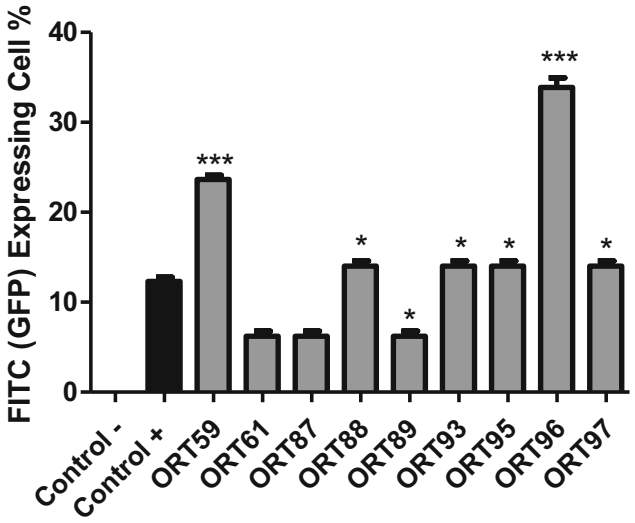


Figure 4. Flow cytometry analysis for FITC (GFP) expressing cell percentages of the transfected cell groups. For transfection studies, the Roche Xtremegene HP protocol was strictly followed with 3:1 of reagent to plasmid volume based ratio. A total of 10^6 RAW 264.7 cells were seeded in 1 mL into 24-well plates and rested overnight at a 37°C 5% CO_2 incubator. ORT series of molecules were dissolved in ethanol and 6 μL of them (12 μg/mL) were used in 1-mL final volume. The pOPINEneo-3C-GFP plasmid was used for the transfection. After 48 h of the transfection, selection process started and continued for 96 h by adding 400 μg/mL of Neomycin into 1 mL of each transfected group's media. Xtremegene HP-GFP expressing plasmid mixture was used as positive control, and in the negative control wells, the plasmid 6 μL ethanol mixture was used. After the flow cytometry analysis, two paired Student's *t* test was applied for the statistical evaluations, $*p < 0.05$, $***p < 0.001$, $N = 3$.

Baulcombe 1999, Vacik et al. 1999, Elbashir et al. 2001, Dass et al. 2002, Pampinella et al. 2002, Brazas and Hagstrom 2005, Grunweller and Hartmann, 2005, Recillas-Targa, 2006, Cuerrier et al. 2007, Yamamoto et al. 2009, Kim and Eberwine 2010). There have been different types of commercially available transfection reagents that enable intracellular gene delivery. These compounds were designed to enable efficient gene delivery into the cells with the least cytotoxicity (Holmen et al. 1995, Carter and Samulski 2000, Ghosh et al. 2000, Kircheis et al. 2001, McGregor et al. 2001, Brazas and Kirschner et al. 2006, Seow and Wood 2009, Slowing et al. 2010, Parelkar et al. 2011, Nimesh et al. 2015). Due to their low performances and high cytotoxicity, new gene delivery reagents should be designed and presented to the field (Holmen et al. 1995, Carter and Samulski 2000, Ghosh et al. 2000, Kircheis et al. 2001, McGregor et al. 2001, Brazas and Kirschner et al. 2006, Seow and Wood 2009, Slowing et al. 2010, Parelkar et al. 2011, Nimesh et al. 2015). In this study, we aimed to synthesize and characterize new gene delivery reagents that would be more efficient and economical to produce while having low cytotoxicity.

Benzimidazoles are heterocyclic compounds that are known for their versatile biological activities (Davidse 1986, Lacey 1990, Katiyar et al. 1994, Khabnadideh et al. 2012, Shin et al. 2009, Díaz-Chiguer et al. 2014, Mobinikhaledi et al., 2014, Ouahrouch et al. 2014, Cevik et al. 2017). These compounds are antimicrobial, antiulcer, antihelmintic, antiinflammatory, anticancer, and antiviral (Davidse 1986, Lacey 1990, Katiyar et al. 1994, Shin et al. 2009, Khabnadideh et al. 2012, Díaz-Chiguer et al. 2014, Mobinikhaledi et al., 2014, Ouahrouch et al. 2014, Cevik et al. 2017). They have been used as efficient fungicides and their mechanism of action is through inhibition of microtubule assembly (Davidse 1986, Lacey 1990, Katiyar et al. 1994, Shin et al. 2009, Khabnadideh et al. 2012, Díaz-Chiguer et al. 2014, Mobinikhaledi et al., 2014, Ouahrouch et al. 2014, Cevik et al. 2017). These compounds prevent microtubule assembly by binding to the subunit molecule, tubulin (Davidse 1986, Lacey 1990, Katiyar et al. 1994, Shin et al. 2009, Khabnadideh et al. 2012, Díaz-Chiguer et al. 2014, Mobinikhaledi et al., 2014, Ouahrouch et al. 2014, Cevik et al. 2017). One advantage of benzimidazole derivatives is that they have higher toxicity against the fungus compared with mammalian cells (Elnima et al., 1981, Shin et al. 2009, Díaz-Chiguer et al., 2014, Ouahrouch et al., 2014, Karaburun et al. 2019). Moreover, these compounds are able to penetrate through the cells which enables their usage as shuttles to carry the DNA inside the cell (Elnima et al., 1981, Davidse 1986, Lacey 1990, Katiyar et al. 1994, Shin et al. 2009, Khabnadideh et al. 2012, Díaz-Chiguer et al. 2014, Mobinikhaledi et al., 2014, Ouahrouch et al., 2014, Cevik et al. 2017, Karaburun et al. 2019). We designed benzimidazole compounds that would have high LogP values as most of the transfection reagents (Holmen et al. 1995, Carter and Samulski 2000, Ghosh et al. 2000, Kircheis et al. 2001, McGregor et al. 2001, Brazas and Kirschner et al. 2006, Seow and Wood, 2009, Slowing et al. 2010, Parelkar et al. 2011, Nimesh et al. 2015). Transfection reagents are lipophilic to pass through the cellular membrane and cationic to engulf the DNA molecule and transfer through the membrane inside the cells (Holmen et al. 1995, Carter and Samulski 2000, Ghosh et al. 2000, Kircheis et al. 2001, McGregor et al. 2001, Brazas and Kirschner et al. 2006, Seow and Wood 2009, Slowing et al. 2010, Parelkar et al. 2011, Nimesh et al. 2015). Benzimidazole derivatives are already able to pass through the cellular membrane (Elnima et al., 1981, Davidse 1986, Lacey 1990, Katiyar et al. 1994, Shin et al. 2009, Khabnadideh et al. 2012, Díaz-Chiguer et al., 2014, Mobinikhaledi et al., 2014, Ouahrouch et al., 2014, Cevik et al. 2017, Karaburun et al. 2019). Designing and synthesizing the derivatives with higher cationic potential would enable efficient transfection of the mammalian cells (Holmen et al. 1995, Carter and Samulski 2000, Ghosh et al. 2000, Kircheis et al. 2001, McGregor et al. 2001, Brazas and Kirschner et al. 2006, Seow and Wood 2009, Slowing et al. 2010, Parelkar et al. 2011, Nimesh et al. 2015).

Xtremegene HP was used as our positive control as a commercially available and widely used transfection reagent in biotechnological applications (Liu et al. 2017). GFP expressing plasmid was used to track the transfection efficiencies directly and quantitatively by flow cytometry analysis (Kim and Eberwine 2010). Compared with Xtremegene HP, benzimidazole derivatives had a couple of fold higher transfection efficiencies. These reagents had comparable cytotoxicities as that of Xtremegene HP on the mammalian macrophages that we used in this study. ORT 59 had 2.5-fold higher efficiency compared with Xtremegene HP product in transfection with similar low cytotoxic levels. Due to chloro substituent and linker length in the structure of ORT 59, it had superior activity compared with other ORT series of molecules. ORT 96 was also highly efficient with 3.5-fold higher transfection efficiency compared with Xtremegene HP, but it had higher but mild cytotoxicity. The reason behind its higher efficiency compared with other ORT molecules might be due to presence of the highest LogP values, linker length, and three chloro substituted groups in its structure. These groups might also lead to higher toxicity as in the cases of ORT 93–97 series of molecules.

Conclusions

Benzimidazole derivatives containing chloro groups had higher transfection efficiency than that of Xtremegene HP. These molecules had mild cytotoxicity levels that were comparable with that of Xtremegene HP. The guidelines of the ROCHE kit were strictly followed in order to prevent any bias towards our molecules. ORT 59 and 96 were the best candidates for the replacement of Xtremegene HP product due to their higher transfection efficiencies as well as similar or mild cytotoxicities (Fig. 4). Most probably, three chloro-substituted (R, R_1, and R_2) chemical groups in their structures and high LogP values lead to greater performances that we observed especially when we compared them with the rest of the ORT series of molecules. In the future studies, new benzimidazole-based derivatives will be designed by taking the structure-activity relationship that we observed in ORT 59 and 96 compounds. These new derivatives will be tested for their transfection efficiencies and cytotoxic side effects both *in vitro* and *in vivo* for transfection and gene therapy applications.

Funding Information This study was financially supported by 2018-2-AP4-2919 BAP Project of Mersin University.

Compliance with Ethical Standards

Conflict of Interest The authors Furkan Ayaz and Oztekin Algul filed a patent application to the Turkish Patent Institute. Other than the patent application, the authors have no conflict of interest to declare.

References

Ayaz F (2018) Ruthenium pyridyl thiocyanate complex increased the production of pro-inflammatory TNFα and IL1β cytokines by the LPS stimulated mammalian macrophages in vitro. Mol Biol Rep 45: 2307–2312

Ayaz F, Ugur N, Ocakoglu K, Ince M (2019) Photo-induced anti-inflammatory activities of chloro substituted subphthalocyanines on the mammalian macrophages in vitro. Photodiagn Photodyn Ther 25: 499–503

Brazas RM, Hagstrom JE (2005) Delivery of small interfering RNA to mammalian cells in culture by using cationic lipid/polymer-based transfection reagents. Methods Enzymol 392:112–124

Carter PJ, Samulski RJ (2000) Adeno-associated viral vectors as gene delivery vehicles. Int J Mol Med 6:17–27

Cevik UA, Saglik BN, Ozkay Y (2017) Synthesis of new fluorobenzimidazole derivatives as an approach towards the discovery of novel intestinal antiseptic drug candidates. Curr Pharm Des 23: 2276–2286

Cuerrier CM, Lebel R, Grandbois M (2007) Single cell transfection using plasmid decorated AFM probes. Biochem Biophys Res Commun 355:632–636

Dass CR, Walker TL, Burton MA (2002) Liposomes containing cationic dimethyl dioctadecyl ammonium bromide formulation, quality control and lipofection efficiency. *Drug Deliv* 9:11–18

Davidse LC (1986) Benzimidazole fungicides: mechanism of action and biological impact. Annu Rev Phytopathol 24:43–65

Díaz-Chiguer DL, Hernández-Luis F, Nogueda-Torres B, Castillo R, Reynoso-Ducoing O, Hernández-Campos A, Ambrosio JR (2014) JVG9, a benzimidazole derivative, alters the surface and cytoskeleton of Trypanosoma cruzi bloodstream trypomastigotes. Mem Inst Oswaldo Cruz 109:757–760

Ehrhardt C, Schmolke M, Matzke A, Knoblauch A, Will C, Wixler V, Ludwing S (2006) Polyethylenimine, a cost-effective transfection reagent. Sign Transduct 6:179–184

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 441:494–468

Elnima EI, Zubair MU, Al-Badr AA (1981) Antibacterial and antifungal activities of benzimidazole and benzoxazole derivatives. Antimicrob Agents Chemother 19:29–32

Gao X, Huang L (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochem Biophys Res Commun 179:280–285

Geisse S, Henke M (2005) Large-scale transient transfection of mammalian cells: A newly emerging attractive option for recombinant protein production. J Struct Funct Genom 6:165–170

Ghosh YK, Visweswariah SS, Bhattacharya S (2000) Nature of linkage between the cationic headgroup and cholesteryl skeleton controls gene transfection efficiency. FEBS Lett 473:341–344

Grunweller A, Hartmann RK (2005) RNA interference as a gene-specific approach for molecular medicine. *Curr Med Chem* 12:3143–3161

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in post transcriptional gene silencing in plants. Science 286:950–952

Harpe AV, Petersen H, Li Y et al (2000) Characterization of commercially available and synthesized polyethylenimines for gene delivery. J Control Release 69:309–322

Holmen SL, Vanbrocklin MW, Eversole RR, Stapleton SR, Ginsberg LC (1995) Efficient lipid-mediated transfection of DNA into primary rat hepatocytes. In Vitro Cell Dev Biol- Animal 31:347–351

Hunt MA, Currie MJ, Robinson BA, Dachs GU (2010) Optimizing transfection of primary human umbilical vein endothelial cells using commercially available chemical transfection reagents. J Biomol Tech 21:66–72

Karaburun AC, Kaya Cavusoglu KB, Acar U et al (2019) Synthesis and antifungal potential of some novel benzimidazole-1,3,4-oxadiazole compounds. Molecules 24:191–205

Katiyar SK, Gordon VR, McLaughlin GL, Edlind TD (1994) Antiprotozoal activities of benzimidazoles and correlations with beta-tubulin sequence. Antimicrob Agents Chemother 38:2086–2090

Khabnadideh S, Rezaei Z, Pakshir K, Zomorodian K, Ghafari N (2012) Synthesis and antifungal activity of benzimidazole, benzotriazole and aminothiazole derivatives. Res Pharm Sci 7:65–72

Kiefer K, Clement J, Garidel P, Peschka-Suss R (2004) Transfection efficiency and cytotoxicity of nonviral gene transfer reagents in human smooth muscle and endothelial cells. Pharm Res 21:1009–1017

Kim TK, Eberwine JH (2010) Mammalian cell transfection: the present and the future. Anal Bioanal Chem 397:3173–3178

Kircheis R, Wightman L, Wagner E (2001) Design and gene delivery activity of modified polyethylenimines. Adv Drug Deliv Rev 31: 348

Kirschner M, Monrose V, Paluch M, Techodamrongsin N, Rethwilm A, Moore JP (2006) Techodamrongsin N,The production of cleaved, trimeric human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein vaccine antigens and infectious pseudoviruses using linear polyethylenimine as a transfection reagent. Protein Expr Purif 48:61–68

Lacey E (1990) Mode of action of benzimidazoles. Parasitol Today 6: 112–115

Liu Y, Fan Z, Li K, Deng F, Xiong Y, Liang M, Ge J (2017) An optimized gene transfection system in WERI-Rb1 cells. Int J Mol Med 40: 801–813

Martinou I, Fernandez PA, Missotten M, White E, Allet B, Sadoul R, Martinou JC (1995) Viralproteins E1B19K and p35 protect sympathetic neurons from cell death induced by NGF deprivation. J Cell Biol 128:201–208

McBain SC, Yiu HHP, El Haj A (2007) Polyethyleneimine functionalized iron oxide nanoparticles as agents for DNA delivery and transfection. J Mater Chem 7:2561–2565

McGregor C, Perrin C, Monck M, Camilleri P, Kirby AJ (2001) Rational Approaches to the Design of Cationic Gemini Surfactants for Gene Delivery. J Am Chem Soc 123:6215–6220

Melissa MF, Hoversten KE, Powers JM, Trobridge GD, Rodgers BD (2013) Genetic manipulation of myoblasts and a novel primary myosatellite cell culture system: comparing and optimizing approaches. FEBS J 280:827–840

Mentese E, Doğan IS, Kahveci B (2013) Green protocol: solvent- and catalyst-free synthesis of benzimidazole derivatives via microwave technique. Chem Heterocycl Compd 49:1136–1140

Mobinikhaledi A, Hamta A, Kalhor M, Shariatzadeh M (2014) Simple synthesis and biological evaluation of some benzimidazoles using sodium hexafluroaluminate, Na_3AlF_6, as an efficient catalyst. Iran J Pharm Res 13:95–101

Moyano DF, Liu Y, Ayaz F, Hou S, Duncan B, Osborne BA, Rotello VM (2016) Immunomodulatory effects of coated gold nanoparticles in LPS-stimulated *in vitro* and *in vivo* murine model systems. Chem 1: 320–327

Nannapaneni D, Gupta AV, Nannapaneni DT, Reddy M (2010) Synthesis, characterization, and biological evaluation of benzimidazole derivatives as potential anxiolytics. J Young Pharm 2:273–279

Nimesh S, Halappanavar S, Kaushik NK, Kumar P (2015) Advances in gene delivery systems. Biomed Res Int 2015:610342–610344

Ouahrouch A, Ighachane H, Taourirte M, Engels JW, Sedra MH, Lazrek HB (2014) Benzimidazole-1,2,3-triazole hybrid molecules: synthesis and evaluation for antibacterial/antifungal activity. Arch Pharm 347:748–755

Pampinella F, Lechardeur D, Zanetti E, MacLachlan I, Benharouga M, Lukacs GL, Vitiello L (2002) Analysis of differential lipofection efficiency in primary and established myoblasts. *Mol Ther* 5:161–169

Parelkar SS, Chan-Seng D, Emrick T (2011) Reconfiguring polylysine architectures for controlling polyplex binding and non-viral transfection. Biomaterial 32:2432–2444

Park H, Yang F, Cho S (2012) Nonviral delivery of genetic medicine for therapeutic angiogenesis. Adv Drug Deliv Rev 64:40–52

Pautz GE, Yang ZY, Wu BY, Gao X, Huang L, Nabel GJ (1993) Immunitherapy of malignancy by *in vivo* gene transfer into tumors. *Proc Natl Acad Sci* 90:4645–4649

Recillas-Targa F (2006) Multiple strategies for gene transfer, expression, knockdown, and chromatin influence in mammalian cell lines and transgenic animals. Mol Biotechnol 34:337–354

Santos AD, Kaïm LE, Grimaud L (2013) Metal-free aerobic oxidation of benzazole derivatives. Org Biomol Chem 11:3282–3287

Seow Y, Wood MJ (2009) Biological gene delivery vehicles: beyond viral vectors. Mol Ther 17:767–777

Shin JM, Sachs G, Cho YM, Garst M (2009) 1-Arylsulfonyl-2-(pyridylmethylsulfinyl) benzimidazoles as new proton pump inhibitor prodrugs. Molecules 14:5247–5280

Simberg D, Hirsch-Lerner D, Nissim R, Barenholz Y (2000) Comparison of different commercially available cationic lipid-based transfection kits. J Liposome Res 10:1–13

Slowing II, Vivero-Escoto JL, Trewyn BG, Lin VSY (2010) Mesoporous silica nanoparticles: structural design and applications. J Mater Chem 20:7924–7937

Vacik J, Dean BS, Zimmer WE, Dean DA (1999) Cell-specific nuclear import of plasmid DNA. *Gene Ther* 6:1006–1014

Wang Y, Sarris K, Sauer DR, Djuric SW (2006) A simple and efficient one step synthesis of benzoxazoles and benzimidazoles from carboxylic acids. Tetrahedron Lett 47:4823–4826

Yamamoto A, Kormann M, Rosenecker J, Rudolph C (2009) Current prospects form RNA gene delivery. Eur J Pharm Biopharm 71: 484–489