*International Journal of Research in Engineering and Science (IJRES)*
*ISSN (Online): 2320-9364, ISSN (Print): 2320-9356*
*www.ijres.org Volume 9 Issue 3 ǁ 2021 ǁ PP. 36-41*

# Preparation of Folic Acid Conjugated Oligonucleotide

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***Abstract***
*Folic acid plays a key role in a number of human metabolic pathways including a nucleic acid synthesis and has been used to develop a number of cancer therapeutic agent. In the meantime, oligonucleotides which exhibit properties other than genomic functions such as specific recognition ability, and bioactivity alteration have been focused in a number of drug delivery systems. It is promising to develop a drug delivery system that integrates the key functions from both folic acid and oligonucleotide. The integration of both molecules is still in challenges and has a way for improvement. In this study, an alternative and feasible conjugation method relying on carbodiimide reaction was investigated. Two coupling reagents were used :1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and dicyclohexylcarbodiimide (DCC). The reaction intermediate was facilitated by the use of N-hydroxysuccinimide (NHS). The conjugation between folic acid and amine modified oligonucleotide was verified by high-performance liquid chromatography (HPLC). The reaction yielded more than one products because folic acid had two reactive sites, and the oligonucleotides could adopt more than one conformation. The key bonding was amide bond. This study is a good model for linking two bioactive molecules via amide coupling reaction based on carbodiimide chemistry.*

***Keywords:****folic acid, oligonucleotides, carbodiimide reaction, NHS, DCC, EDC*

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Date of Submission: 27-02-2021 Date of acceptance: 12-03-2021

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## I. INTRODUCTION

Folic acid (FA) which is known as vitamin B9 can be soluble in water in a form of folate ion. Its structure composed of the pteridine ring, para-amino benzoic acid, and glutamic acid with an overall molecular weight of 441 Da as depicted in Fig 1. FA plays a key role in a number of human metabolic pathways including a nucleic acid synthesis [1], because it is an essential carrier for the biosynthesis of purine and thymidine. These two molecules are building block for nucleic acid synthesis, mainly undergone by methylation. The nucleic acid further facilitates amino acid metabolism which is mandatory for healthy fetus development [2]. FA is internalized into cells via receptor-mediated endocytosis when it binds to folate receptor (FR) at γ-carboxyl group with an approximate binding affinity of 1 nM [3]. FR, a glycosyl-phosphatidylinositol-anchored protein, is overexpressed on membranes of many human cancer cells such as breast, brain, kidney, lung, and ovarian cancers)[4-7], while it has less expression in normal tissues [8]. Therefore, both FA and FR have drawn much attention in the development of cancer-specific markers and cancer targeted drugs [9, 10]. In the literature, FA was conjugated to boron nitride nanospheres for developing drug delivery system for doxorubicin (Dox). This delivery system facilitated cellular uptake of Dox which mediated by the FA receptor. The delivery system provided more efficient uptake than the uptake of free Dox [11]. This showed promising strategy for development alternative treatment for cancers and other diseases. Currently, four types of FA-drug conjugates have been undergone clinical trials for the treatment of cancer: Vintafolide (EC145) [12, 13], EC0225[14], BMS-753493[15], and EC0489[16].

Oligonucleotides are short, single- or double-stranded DNA or RNA molecules with the length about 8-50 nucleotides [17]. They have been categorized into different types based on their biological activity such as antisense oligonucleotides (ASO) [18], aptamers [19], spice-switching oligonucleotides [20], siRNA[21], and miRNA [22]. For example, AZD9150 which was an ASO was able to decrease the expression of transcription 3 (STAT3) in lung cancer. It shows a promise on antitumor activity for several cancers in phase I clinical trials[23]. Pegaptanib, a vascular endothelial growth factor (VEGF)-specific aptamer, was approved by the Food and Drug Administration (FDA) for applying in the treatment of neovascular (wet) age-related macular degeneration (AMD) [24]. MiR-506, a miRNA, which induced cell cycle arrest at the G1/S transition and promoted apoptosis of cervical cancer cells [25]. Basically, the oligonucleotide structure possesses hydrophilicity, anionic charge and enzymatic degradability [26] which make them exhibit poor pharmacokinetic for nucleic acid-based drugs [27], cellular uptake difficultly [28], deficient stability and possibly off-target effects [29]. To overcome those issues, the oligonucleotides have been incorporated into a number of materials and/or binding ligand such as nanoparticle, polymer, liposome, carbohydrate, protein, cholesterol, and

tocopherol [18, 30]. Conjugation of oligonucleotides with FA via simple reaction have been investigated for improving specificity of cancer applications such as, folate-conjugated microRNA [31]and folate-conjugated Luc siRNA [32]. However, conjugation efficiency and cost effectiveness remain challenges.

In this study, carbodiimide reaction between FA and an oligonucleotide named as folic linker (FL) were studied as an alternative and feasible conjugation method for synthesizing folic acid conjugated oligonucleotide (FA-FL conjugate). Two coupling reagents were investigated: 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and dicyclohexylcarbodiimide (DCC). The conceptual reaction is presented in Fig.1. The reaction products were followed by high-performance liquid chromatography (HPLC).

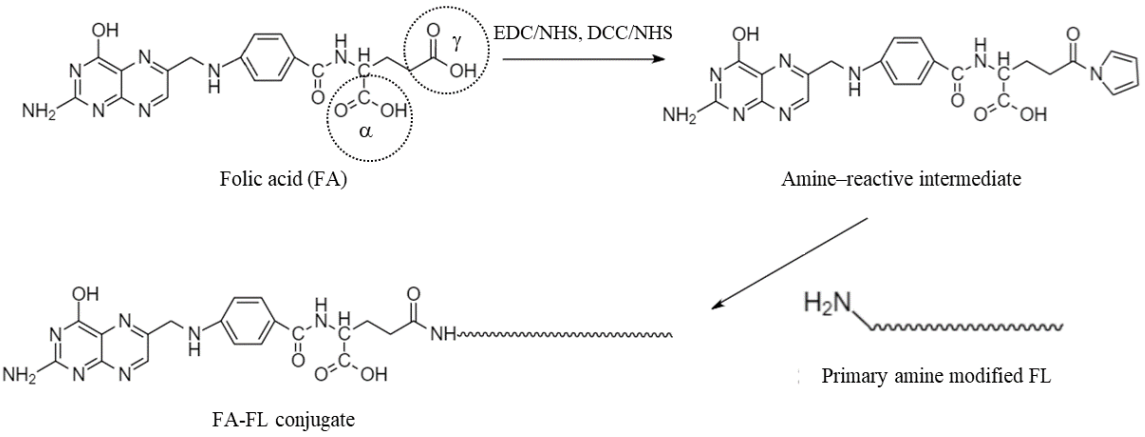


**Figure 1: Synthesis of FA-FL conjugate.**

## II. MATERIALS AND METHODS

### 2.1 Reagents

Folic acid (FA), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) dicyclohexylcarbodiimide (DCC) N-hydroxysuccinimide (NHS), anhydrous dimethyl sulfoxide (DMSO), Triethylaluminium acetate and Acetonitrile were purchased from Sigma-Aldrich (USA). Oligonucleotide (FL) was purchased from Integrated DNA Technologies (USA).

### 2.2 Verification of folic linker (FL) by HPLC

HPLC condition for characterizing FL was optimized, by injecting the FL at different concentrations to the instrument.FL with concentrations of 1, 5, 10, 20 μM was used to test the condition developed by Tang et al., 2017[33]. The key instruments composed of a 5 μm inertsil ODS-3 column (4.6 x 250mm) and diode array detector setting a detection wavelength at 260 nm. Before injection, all solutions including mobile phase were filtered through 0.22 μm nylon membrane. Then all solutions were injected at a flow rate of 1.0 mL/min and the injection volume was set at 20 μl. The elution was carried out by a mixture of 10 mM triethylaluminium acetate (A) and 100% acetonitrile (B) with gradient conditions: 2.0% of B for 0.5 min, 2.0 to 17.4% of B over 11 min, 17.4 to 60.0% of B over 0.1 min, 60.0% of B for 2 min, 60.0 to 2.0% of B over 0.1 min, and 2.0% B over 3.3 min.

### 2.3 Synthesis of FA-FL conjugate using EDC/NHS as coupling reagent

FA was chemically linked to FL via carbodiimide reaction using EDC/NHS as coupling agent. FA was dissolved in DMSO with a concentration of 0.05 M. Separately, NHS and EDC were dissolved in DMSO with a concentration of 0.8 and 4 M, respectively. Carboxylic activation was performed by mixing FA, EDC, and NHS. The mixture was incubated at room temperature for 30 minutes. After that, primary amine modified FL with a concentration of 1 mM was added into the activated mixture. The carbodiimide reaction was further carried out for overnight. The reaction was characterized by HPLC (Shimazu, Japan).

### 2.4 Synthesis of FA-FL conjugate using DDC/NHS as coupling reagent

FA was dissolved in DMSO at a concentration of 0.0706 M. Then an equal molar of NHS and DCC was dissolved in DMSO at a concentration of 0.04 M. The coupling agent was mixed with FA solution for the formation of an amine reactive intermediate. The reaction was incubated for 4 hours at room temperature and dark condition. Next, FL bearing primary amine at 3' end with a concentration of 10 μM was added to the mixture for amide bond formation. The reaction was continuous incubated overnight. The reaction was then filtered to remove by-product using 0.25μm cellulose membrane. FA-FL conjugate was confirmed by HPLC.

### 2.5 Characterizations of FA-FL conjugate

HPLC was used for determining the products of conjugation between FA and FL using the same instrument setup and conditionas in the previous section.All solutions were filtered through 0.22 μm nylon membrane. The injection volume was 20 μl. The gradient elution was set at a flow rate of 1.0 mL/min.The mobile phase composed of 10 mM of triethylaluminium acetate and acetonitrile.

## III. RESULT AND DISCUSSION

### 3.1 Optimization of HPLC condition for FL detection

HPLC condition was required to be optimized for the detection of FL. The FL at different concentrations was injected into HPLC, and the chromatogram was presented in Fig 2 A&B. The peak that corresponded to FL was at 23.92533, 22.90133, 22.848 and 22.848 minutes for 1, 5, 10 and 20μM FL, respectively. The detected intensity was dose dependent as depicted in Fig 2C.This result suggested that FL which is an oligonucleotide sequences was able to be detected by HPLC using the studied condition, and HPLC was a capable technique for characterizing the reaction between FA and FL.

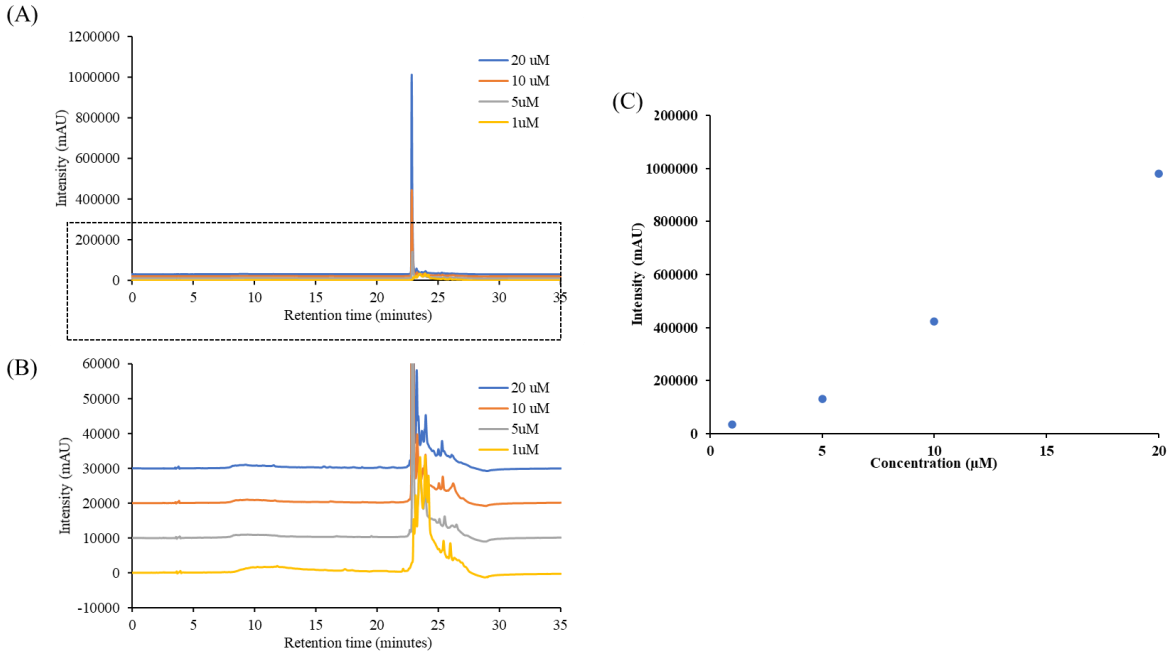


**Figure 2: (A) overall and (B) zoomed in chromatogram of FL. The dash box indicates the area of the chromatogram that is magnified. 1 μM (yellow line), 5 μM (gray line), 10 μM (orangeline), and 20 μM (blue line). (C) Peak intensity of FL at differentconcentrations.**

### 3.2 Synthesis of FA-FL conjugate using EDC/NHS coupling reagent

FA had γ-carboxylic acid that was selectively activated by EDC/NHS resulting amine-reactive NHS ester. This ester was a reactive intermediate which reacted with primary amine available on FL to form amide bonding [34].

The FA-FL conjugate prepared by EDC/NHS was examined by HPLC as shown in Fig 3. The chromatogram of the reaction mixture showed that there were four peaks at retention times of 14.635, 15.093, 15.861, and 20.469 minutes (red arrow) with intensity as 9504, 9072, 13751 and 7308 mAU that did not appear in the chromatogram of those initial reactants: FA, FL, and EDC/NHS. This result suggested that these four peaks were different FA-FL conjugates. The possible explanations were as follows. FA contained two reactive carboxylic groups as indicated in Fig 1., which could yield different conjugation products. Beside FL was an oligonucleotide which could have different conformation leading to different partition ability in the chromatography [35].

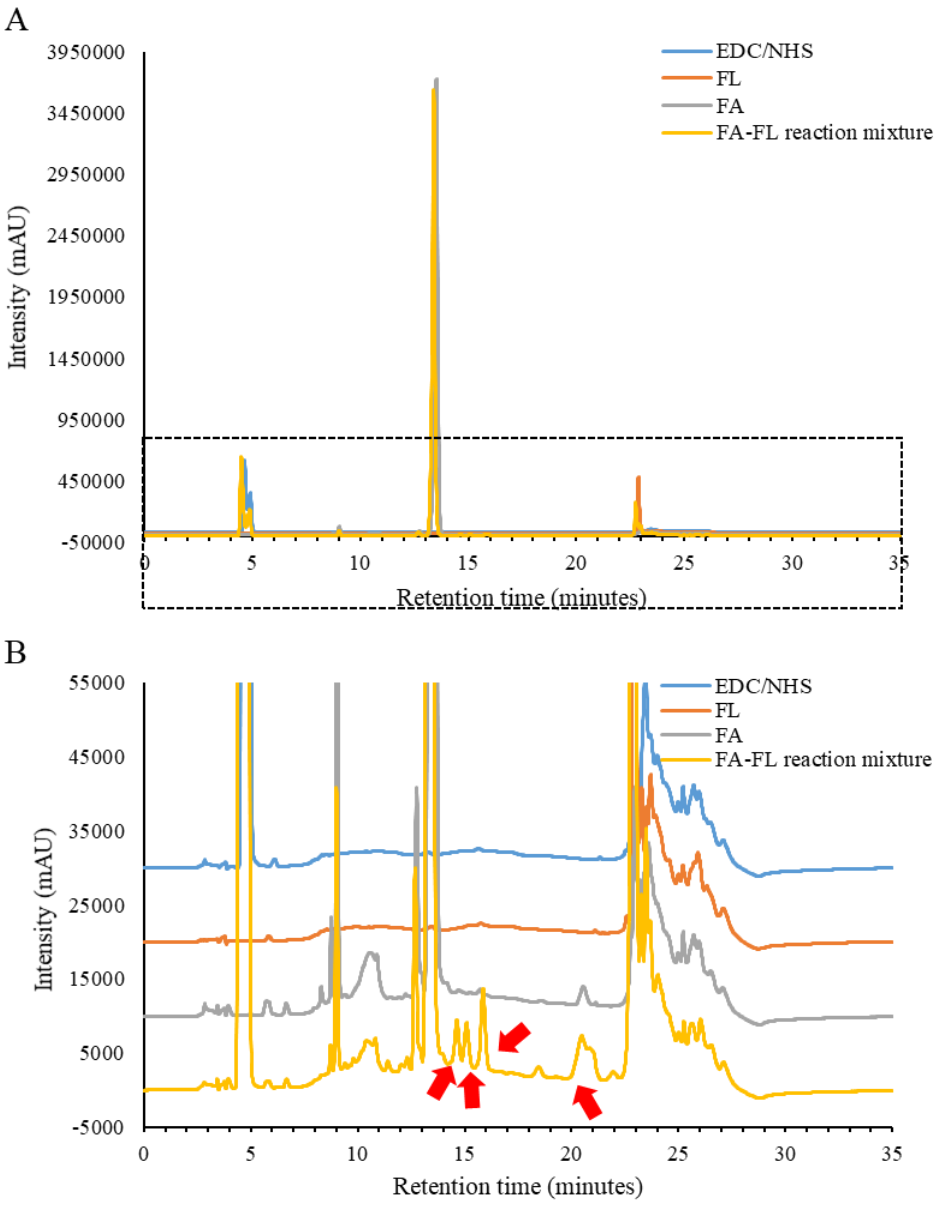


**Figure 3: (A) overall and (B) zoomed in chromatogram of FA-FL conjugate using EDC/NHS as coupling agent. The dash box indicates the area of the chromatogram that is magnified. Reaction mixture (yellow line), FA (gray line), FL (orangeline), and EDC/NHS mixture (blue line). Red arrows indicate reaction products.**

### 3.3 Synthesis of FA-FL conjugate using DCC/NHS as coupling reagent

DCC/NHS, an alternative coupling system, was used to activate the γ-carboxylic acid of FA. The activated product was further forming the amide bond with primary amine modified FL. HPLC results showed that three peaks at retention times of 14.421, 15.605, and 20.576 minutes (purple arrow) with intensity as 3734, 4519 and 6277 mAU were assigned as the reaction products since they do not appear in the chromatogram of the initial reactants (Fig 4.). This result also informed that FA-FL conjugates were a combination of molecules yielded by DCC. DCC is a cyclic and water insoluble compound [36] which possibly provides a more reactive selectivity therefore the coupling products was less complex than the reaction that uses EDC as a coupling agent.

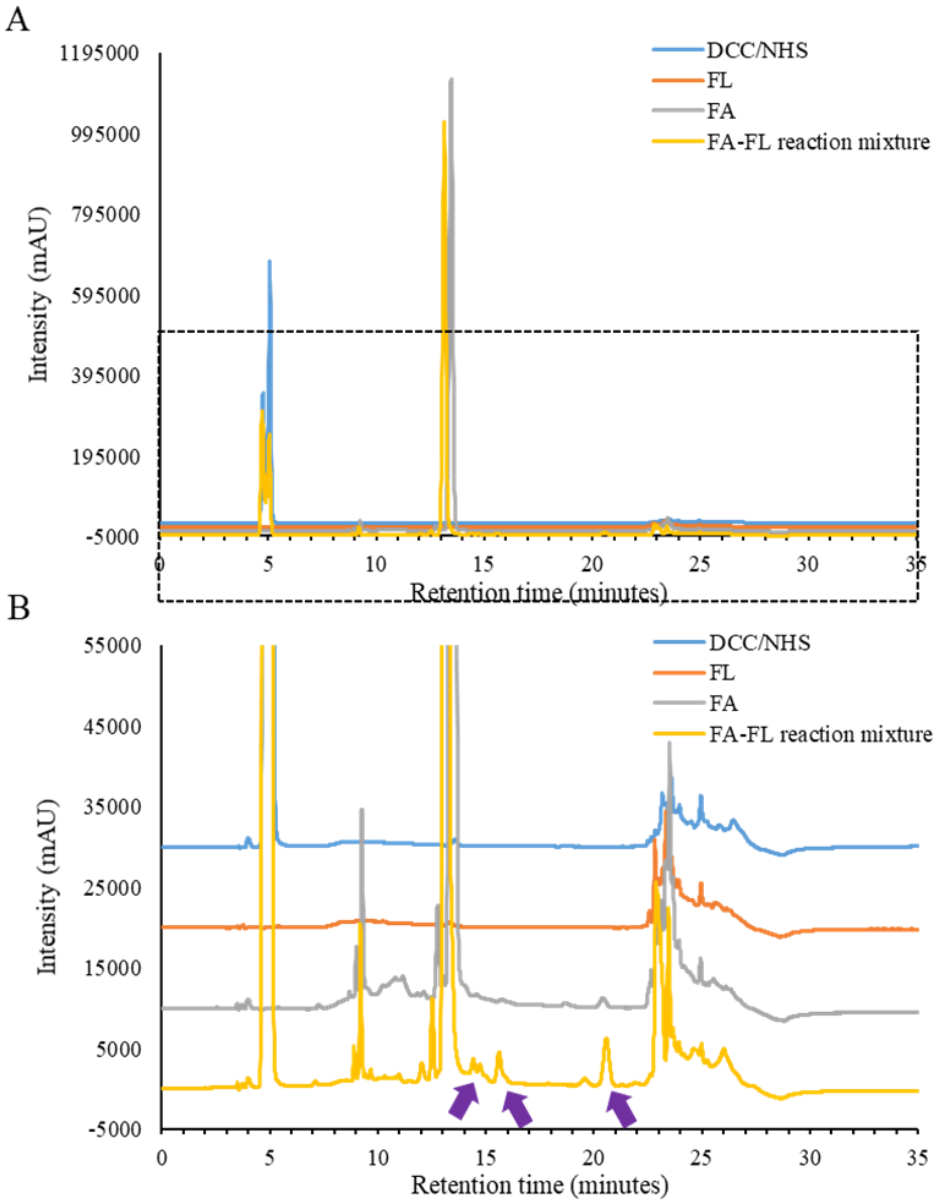


**Figure 4: (A) overall and (B) zoomed in chromatogram of FA-FL conjugate using DCC/NHS as coupling agent. The dash box indicates the area of the chromatogram that is magnified. Reaction mixture (yellow line), FA (gray line), FL (orangeline), and DCC/NHS mixture (blue line). Purple arrows indicate reaction products.**

## IV. CONCLUSION

In conclusion, FA-FL conjugate was synthesized using two coupling systems: EDC/NHS, and DCC/NHS. HPLC chromatograms indicated that there were more than one reaction products formed due to the availability of carboxylic group in FA and the different conformation of FL. The amide bond was the linkage between and FA and FL. The amide coupling reaction based on carbodiimide chemistry is an alternative strategy to link a nucleic acid moiety to a bioactive molecule.

## REFERENCES

[1]. Emsley J. (1998) "Molecules at an Exhibition" Oxford, U.K., Oxford University Press, Pp. 60-62.
[2]. Choi S-W, Mason J. (2000) "Folate and Carcinogenesis: An Integrated Scheme" J Nutr, Vol. 133, No., Pp.3748-3753.
[3]. Salter DN, Scott KJ, Slade H, Andrews P. (1981) "The preparation and properties of folate-binding protein from cow's milk" Biochem J, Vol. 193, No. 2, Pp.469-476.
[4]. Hartmann L, Keeney G, Lingle W, Christianson T, Varghese B, Hillman D, et al. (2007) "Folate receptor overexpression is associated with poor outcome in breast cancer" Int J Cancer, Vol. 121, No., Pp.938-942.

[5]. Nunez MI, Behrens C, Woods DM, Lin H, Suraokar M, Kadara H, et al. (2012) "High expression of folate receptor alpha in lung cancer correlates with adenocarcinoma histology and EGFR [corrected] mutation" J Thorac Oncol, Vol. 7, No. 5, Pp.833-840.

[6]. Parker N, Turk MJ, Westrick E, Lewis JD, Low PS, Leamon CP. (2005) "Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay" Analytical biochemistry, Vol. 338, No. 2, Pp.284-293.

[7]. Toffoli G, Cernigoi C, Russo A, Gallo A, Bagnoli M, Boiocchi M. (1997) "Overexpression of folate binding protein in ovarian cancers" Int J Cancer, Vol. 74, No. 2, Pp.193-198.

[8]. Sudimack J, Lee RJ. (2000) "Targeted drug delivery via the folate receptor" Adv Drug Deliv Rev, Vol. 41, No. 2, Pp.147-162.

[9]. Low PS, Antony AC. (2004) "Folate receptor-targeted drugs for cancer and inflammatory diseases" Adv Drug Deliv Rev, Vol. 56, No. 8, Pp.1055-1058.

[10]. Chen C, Ke J, Zhou XE, Yi W, Brunzelle JS, Li J, et al. (2013) "Structural basis for molecular recognition of folic acid by folate receptors" Nature, Vol. 500, No. 7463, Pp.486-489.

[11]. Feng S, Zhang H, Yan T, Huang D, Zhi C, Nakanishi H, et al. (2016) "Folate-conjugated boron nitride nanospheres for targeted delivery of anticancer drugs" Int J Nanomedicine, Vol. 11, No., Pp.4573-4582.

[12]. Leamon CP, Reddy JA, Vlahov IR, Westrick E, Dawson A, Dorton R, et al. (2007) "Preclinical antitumor activity of a novel folate-targeted dual drug conjugate" Mol Pharm, Vol. 4, No. 5, Pp.659-667.

[13]. Reddy JA, Dorton R, Westrick E, Dawson A, Smith T, Xu LC, et al. (2007) "Preclinical evaluation of EC145, a folate-vinca alkaloid conjugate" Cancer res, Vol. 67, No. 9, Pp.4434-4442.

[14]. Sharma S, Sausville EA, LoRusso P, Vogelzang NJ, Samlowski WE, Carter J, et al. (2010) "A phase I study of EC0225 administered weeks 1 and 2 of a 4-week cycle" J Clin Oncol, Vol. 28, No. 15, Pp.3082-3082.

[15]. Peethambaram PP, Hartmann LC, Jonker DJ, de Jonge M, Plummer ER, Martin L, et al. (2015) "A phase I pharmacokinetic and safety analysis of epothilone folate (BMS-753493), a folate receptor targeted chemotherapeutic agent in humans with advanced solid tumors" Invest New Drugs, Vol. 33, No. 2, Pp.321-331.

[16]. Leamon CP, Reddy JA, Klein PJ, Vlahov IR, Dorton R, Bloomfield A, et al. (2011) "Reducing undesirable hepatic clearance of a tumor-targeted vinca alkaloid via novel saccharopeptidic modifications" J Pharmacol Exp Ther, Vol. 336, No. 2, Pp.336-343.

[17]. Lakhia R, Mishra A, Patel V. (2019) "Manipulation of renal gene expression using oligonucleotides" Methods Cell Biol, Vol. 154, No., Pp.109-120.

[18]. Yamamoto T, Nakatani M, Narukawa K, Obika S. (2011) "Antisense drug discovery and development" Future Med Chem, Vol. 3, No. 3, Pp.339-365.

[19]. Ellington AD, Szostak JW. (1990) "In vitro selection of RNA molecules that bind specific ligands" Nature, Vol. 346, No. 6287, Pp.818-822.

[20]. Bauman J, Jearawiriyapaisarn N, Kole R. (2009) "Therapeutic potential of splice-switching oligonucleotides" Oligonucleotides, Vol. 19, No. 1, Pp.1-13.

[21]. Wang Z, Rao DD, Senzer N, Nemunaitis J. (2011) "RNA interference and cancer therapy" Pharm Res, Vol. 28, No. 12, Pp.2983-2995.

[22]. Yeung ML, Jeang KT. (2011) "MicroRNAs and cancer therapeutics" Pharm Res, Vol. 28, No. 12, Pp.3043-3049.

[23]. Hong D, Kurzrock R, Kim Y, Woessner R, Younes A, Nemunaitis J, et al. (2015) "AZD9150, a next-generation antisense oligonucleotide inhibitor of STAT3 with early evidence of clinical activity in lymphoma and lung cancer" Sci Transl Med, Vol. 7, No. 314, Pp.314ra185.

[24]. Gragoudas ES, Adamis AP, Cunningham ET, Jr., Feinsod M, Guyer DR. (2004) "Pegaptanib for neovascular age-related macular degeneration" N Engl J Med, Vol. 351, No. 27, Pp.2805-2816.

[25]. Wen SY, Lin Y, Yu YQ, Cao SJ, Zhang R, Yang XM, et al. (2015) "miR-506 acts as a tumor suppressor by directly targeting the hedgehog pathway transcription factor Gli3 in human cervical cancer" Oncogene, Vol. 34, No. 6, Pp.717-725.

[26]. Winkler J. (2013) "Oligonucleotide conjugates for therapeutic applications" Ther Deliv, Vol. 4, No. 7, Pp.791-809.

[27]. Dirin M, Winkler J. (2013) "Influence of diverse chemical modifications on the ADME characteristics and toxicology of antisense oligonucleotides" Expert Opin Biol Ther, Vol. 13, No. 6, Pp.875-888.

[28]. Koller E, Vincent TM, Chappell A, De S, Manoharan M, Bennett CF. (2011) "Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes Nucleic Acids Res, Vol. 39, No. 11, Pp.4795-4807.

[29]. Winkler J, Stessl M, Amartey J, Noe CR. (2010) "Off-Target Effects Related to the Phosphorothioate Modification of Nucleic Acids" ChemMedChem, Vol. 5, No. 8, Pp.1344-1352.

[30]. Gomes-da-Silva LC, Fonseca NA, Moura V, Pedroso de Lima MC, Simões S, Moreira JN. (2012) "Lipid-based nanoparticles for siRNA delivery in cancer therapy: paradigms and challenges" Acc Chem Res, Vol. 45, No. 7, Pp.1163-1171.

[31]. Orellana EA, Tenneti S, Rangasamy L, Lyle LT, Low PS, Kasinski AL. (2017) "FolamiRs: Ligand-targeted, vehicle-free delivery of microRNAs for the treatment of cancer" Sci Transl Med, Vol. 9, No. 401, Pp.eaam9327.

[32]. Cheung A, Bax HJ, Josephs DH, Ilieva KM, Pellizzari G, Opzoomer J, et al. (2016) "Targeting folate receptor alpha for cancer treatment" Oncotarget, Vol. 7, No. 32, Pp.52553-52574.

[33]. Tang Q, Cai A, Bian K, Chen F, Delaney JC, Adusumalli S, et al. (2017) "Characterization of Byproducts from Chemical Syntheses of Oligonucleotides Containing 1-Methyladenine and 3-Methylcytosine" ACS Omega, Vol. 2, No. 11, Pp.8205-8212.

[34]. Kim SL, Jeong HJ, Kim EM, Lee CM, Kwon TH, Sohn MH. (2007) "Folate receptor targeted imaging using poly (ethylene glycol)-folate: in vitro and in vivo studies" J Korean Med Sci Vol. 22, No. 3, Pp.405-411.

[35]. Largy E, Mergny J-L. (2014) "Shape matters: size-exclusion HPLC for the study of nucleic acid structural polymorphism" Nucleic acids res, Vol. 42, No. 19, Pp.e149-e149.

[36]. Kvasnica M. (2007) "Dicyclohexylcarbodiimide (DCC)" Synlett, Vol. 2007, No. 14, Pp.2306-2307.