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Polymer-linked Gemcitabine Prodrugs: Synthesis, *In-vitro* Evaluation and Comparative Studies for targeted delivery to Colon.

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ABSTRACT
Modern analytical techniques were used to create colon-specific azo-based polymeric drug conjugates (6–14) and describe them. To confirm the release pattern of the medication (Gemcitabine) from the polymeric backbone, in vitro stability tests were conducted in simulated acidic (pH 1.2) and alkaline (pH 7.4) environments. Additionally, the in-vitro release behavior of each produced polymer (polyphosphazene and chitosan) linked drug conjugate was assessed in the presence of rat caecal material. According to the findings, rat caecal content displayed a maximum polymeric drug conjugate (13) release of 86.8%. Additionally, a comparison study between polyphosphazene and chitosan was conducted, and the results showed that polyphosphazene-based conjugates performed better than chitosan-based conjugates. Therefore, pharmacological conjugates are based on polyphosphazene azo. Therefore, polyphosphazene azo-based drug conjugates will be considered a potential candidate for further study in the future.
Keywords: *polyphosphazene; prodrug; conjugate; gemcitabine*

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INTRODUCTION

The leading cause of death worldwide is cancer. When cells suffer genetic damage that renders them unable to respond to normal tissue regulation, cancer is triggered. According to several studies [12, 4, 18, 29, 40], improvements in early detection, surgery, radiotherapy, and chemotherapy have been shown to improve curability and survival. According to http://cancer.about.com, skin cancer, thyroid cancer, prostate cancer, breast cancer, colon cancer, lung cancer, leukemia, and bladder cancer are the most prevalent types of cancer. Colorectal cancer is the third most frequent cause of mortality in this nation overall [36].

For a range of local treatments, including ulcerative colitis, cirrhosis disease, amoebiasis, and colon cancer, targeted drug administration into the colon is extremely desirable [3]. It was discovered that a colonic targeted approach was successful in reducing undetermined adverse effects [9-13]. The prodrug technique is the most often used approach among the many colon drug delivery system options. The prodrug approach for colon cancer incorporates covalent bonding between the drug and its carrier, ensuring that after oral administration, the moiety stays intact both before and after reaching the colon. In the azo bond conjugate prodrug method, the drug is joined to a carrier by an azo bond. The colon's azo bond, that is intact in the upper Gastrointestinal tract, is broken by the microflora's azo reductase [32]. The colon's microflora is composed primarily of anaerobic bacteria, such as eubacteria, Bacteroides, bifidobacteria, enterobacteria, clostridia, enterococci, etc., and is in the range of 1011–1012 CFU/ml [35]. A large variety of other enzymes, including glucuronidase, xlyosidase, nitroreductase, and azoreductase, are also produced by microflora [26]. Biodegradable polymers, such as polyesters, polyorthoesters, polyanhydrides, polyacetals, polycarbonates, and polyphosphazene [30-32], and chitosan, play a significant role in drug delivery systems. Biodegradable polyphosphazenes, a family of relatively recent biomaterials, are composed of alternating nitrogen as well as phosphorous atoms containing organic side groups connected to the phosphorous atom. [1,2]. Numerous biological uses of polyphosphazene exist, including those for chemotherapeutic medicines [24, 29, 8], growth factors [21, 22, 24], DNA [16, 36, 37], and proteins [and vaccines [5,6,17]. A polysaccharide called chitosan is derived from the shells of crustaceans including shrimp, crabs, and others [34]. Due to its safety, it is regarded as a valuable material for oral medication delivery systems because it is biocompatible and biodegradable [19].

Chitosan is used widely in the pharmaceutical industry as a disintegrant, a drug carrier in microparticle systems, a binder in wet granulation, a site-specific drug delivery system (for example, to stomach or colon), a slow-release agent for tablets and granules, and a binder in wet granulation. Stavroula *et al*. [34] Using a targeted polymeric prodrug design method is a fresh way to distribute drugs effectively and strategically. This method uses an azo bond to covalently attach the drug molecule to the polymeric backbone. It depends on many variables, including the makeup of the polymeric backbone and the drug carrier [14]. In this work, gemcitabine (1) is used as the model medication. Deoxycytidine analogue gemcitabine is effective in treating human leukemia [11, 13]. Numerous experimental solid tumors, including small cell lung cancer, non-small cell lung cancer, pancreatic and ovarian cancer, are responsive to gemcitabine. Combinations are successful against a variety of different solid tumors, including ovarian cancer, bladder cancer, gastric cancer, and, to a lesser extent, stomach and esophageal cancer [20]. Gemcitabine is not regarded as a safe medicine due to a variety of negative effects and its rapid metabolism. In the treatment of colorectal cancer, it has frequently been used in conjunction with the drug 5-fluorouracil (5-FU) [15]. An azo-derived prodrug of gemcitabine (3) has already been described. The results revealed that the azo-based gemcitabine prodrug demonstrated improved stability in the upper GIT tract and released 60–70% of its dose in the rat caecal content. As our research team develops an azo-based prodrug strategy to deliver anticancer medicines to the colon, one of the target sites. Based on the aforementioned results, we decided to compare the advantages of an azo-based prodrug strategy with those of biodegradable polymers like polyphosphazene and chitosan. So, in the current communication, colon-specific azo-based polyphosphazene-linked and chitosan-linked prodrugs of gemcitabine have been created and assessed. To determine whether polymer shows superior release at the target site, a comparison study of polyphosphazene drug conjugate and chitosan drug conjugate was conducted.

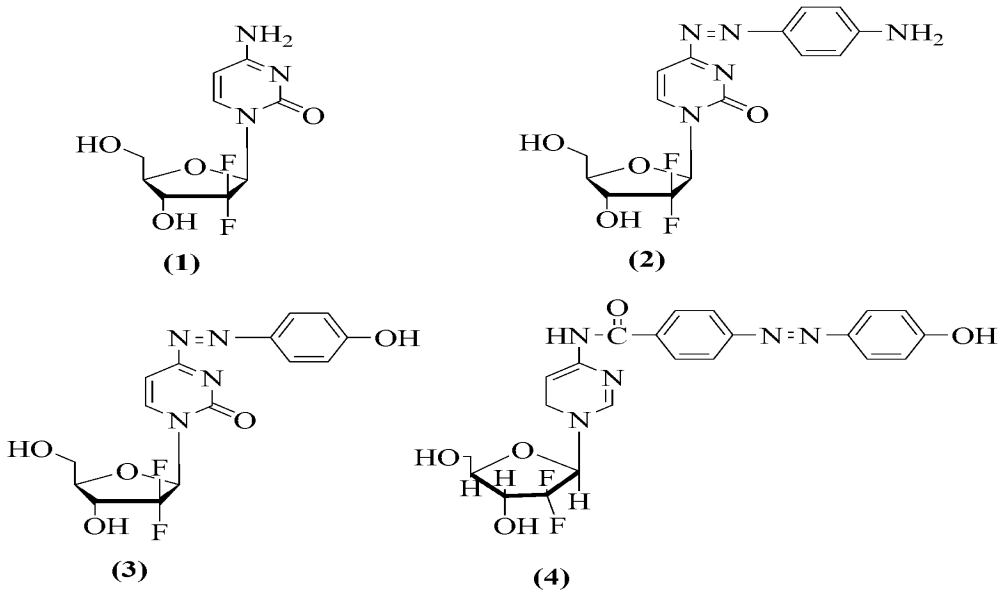


Fig.1 2D Chemical Structures of Gemcitabine (**1**), Azo Prodrugs of gemcitabine (**2**), (**3**), (**4**)

All of the chemicals and reagents used came from Sigma Aldrich, Loba, and CDH in India. Before use, the solvents were distilled per accepted practice. Thin layer chromatography (TLC) was used to verify that the reaction was completed. Plates with silica gel Precoated sheets with Kiesel gel 0.25 mm, 60G F254 employed for TLC and iodine vapors and ultraviolet light as the visualizing agent to visualize spots. Using KBr pellets and a Perkin-Elmer 1600 FTIR spectrometer, the IR spectra (KBr) cm-1 were collected. Tetramethylsilane was used as the internal reference to record 1H NMR spectra (δ, ppm) in DMSO-d6 solutions using a Bruker Avance II 400 spectrometer operating at 400 MHz. From Arch Pharmalabs Ltd in Thane, Maharashtra, gemcitabine was purchased.

MATERIALS AND METHODS

Experimental procedure for the synthesis of azo-based prodrugs of gemcitabine (2-4)

Synthesis of an azo-based prodrug of gemcitabine (2,3)

The azo-based prodrugs of gemcitabine (**2,3**) were synthesized as per the literature procedure [28, 29].

Synthesis of modified polyphosphazene drug conjugates (6-13)

Generic steps for the preparation of gemcitabine substituted polyphosphazene-linked azo prodrug (6-9)

Poly(dichloro)phosphazene **(5)** (80 mg, 0.0006 moles) was dissolved in THF (20 ml) and was added to ethanol (0.04 ml, 0.0006 moles) for **(6),** *tert*-butanol (0.06ml, 0.0006 moles) for **(7),** 4-chloroaniline (87mg, 0.0006 moles) for **(8),** 3-nitroaniline (94mg, 0.0006 moles) for **(9)**, triethylamine (0.17ml) in THF (5.0ml) was added under nitrogen over a period of 30 min. The reaction was continuously refluxed for 24 hrs. Then to this a solution 4-[(4-Aminophenylazo)-1-(3,3-difluoro-4-hydroxy-5-hydroxymethyl tetrahydrofuran-2-yl)]-pyrimidin-2-one (**2**) (0.250mg,0.0006 moles)in THF(10ml). The reaction was further processed as per the literature procedure [29].

Poly(ethoxy)[4-(4-Aminophen-1-yldiazo)-1-(3,3-Difluoro-Tetrahydro-4-Hydroxy-5-(Hydroxymethyl) furan-2-yl)-Pyrimidin-2-one]Phosphazene. (6)

I.R.(KBr cm^{-1}): 1638(N=N), 1654(CON-), 1598(C=N) ,1618(C=C), 1279(P=N), 1139(C-F), 1306(C-N) , 3475(NH) , 3405(OH).^{1}H-NMR (400MHz, $CDCl_3$, δ, TMS=0, *J*=Hz): 7.3(d, 1H, pyrimidine, *J*=8.4), 7.2(d, 1H, Ar-H, *J*=2.1), 7.1(d, 1H, Ar-H, *J*=2.1), 6.6(d, 1H, Ar-H, *J*=2.7) 6.52(d, 1H, Ar-H, *J*=2.7), 6.4(s, 1H, ribose), 4.7(d, 1H, pyrimidine, *J*=8.4) , 4.2(s, 1H, ribose), 4.1(br, 1H,NH), 4.01(m,2H, CH_2), 3.8(s, 1H, ribose), 3.7(d, 2H, CH_2), 3.4(s, 1H, OH), 3.0(s, 1H, CH), 1.29(t,3H,CH_3).^{31}P- NMR ($CDCl_3$) : δ -16.5 ppm [s ; rel. to $(Ph)_3P$]

Poly(t-Butoxy)[4-(4-aminophen-1-yldiazo)-1-(3,3-Difluoro-Tetrahydro-4-Hydroxy-5-(Hydroxymethyl)furan-2-yl)-Pyrimidin-2-one] Phosphazene. (7)

I.R.(KBr, cm^{-1}): 1633(N=N), 1654(CON-), 1598(C=N) ,1618(C=C), 1238(P=N), 1139(C-F), 1306(C-N) , 3450(NH), 3390(OH).^{1}H-NMR (400MHz, $CDCl_3$, δ, TMS=0, *J*=Hz): 7.3(d, 1H, pyrimidine, *J*=8.4), 7.4(d, 1H, Ar-H, *J*=2.1), 6.9(d, 1H, Ar-H, *J*=2.1), 6.5(d, 1H, Ar-H, *J*=2.7) 6.52(d, 1H, Ar-H, *J*=2.7), 6.4(s, 1H, ribose), 4.7(d, 1H, pyrimidine, *J*=8.4) , 4.2(s, 1H, ribose), 4.1(br, 1H,NH), 3.8(s, 1H, ribose), 3.7(d, 2H, CH_2), 3.4(s, 1H, OH), 3.0(s, 1H, CH), 1.19 (t, 9H,CH_3).^{31}P- NMR ($CDCl_3$) : δ -17.9 ppm [s ; rel. to $(Ph)_3P$].

Poly(4-chloroanilino)[4-(4-Aminophen-1-yldiazo)-1-(3,3-Difluoro-Tetrahydro-4-Hydroxy-5-(Hydroxymethyl)Furan-2-yl)-Pyrimidin-2-one] Phosphazene (8)

I.R.(KBr, ν cm^{-1}): 1630(N=N), 1644(CON-), 1570(C=N) ,1625(C=C), 1232(P=N), 1135 (C-F), 1306(C-N) , 3470(NH) , 3400(OH).^{1}H-NMR (400MHz, $CDCl_3$, δ, TMS=0, *J*=Hz): 7.71(d, 2H, Ar-H), 7.49(d, 2H, Ar-H),7.2(d, 1H, pyrimidine, *J*=8.4), 7.6(d, 1H, Ar-H, *J*=2.1), 7.0(d, 1H, Ar-H, *J*=2.1), , 6.95-7.01 (m, 4H, Ar-H), 6.3(d, 1H, Ar-H, *J*=2.7) 6.4(d, 1H, Ar-H, *J*=2.7), 6.2(s, 1H,ribose), 4.6(d, 1H, Pyrimidine, *J*=8.4) , 4.4(s, 1H, ribose), 3.9(br, 1H,NH), 3.7(s, 1H, ribose), 3.5(d, 2H, CH_2), 3.2(s, 1H, OH), 2.8(s, 1H, CH). ^{31}P- NMR ($CDCl_3$) : δ -15.7 ppm [s ; rel. to $(Ph)_3P$]

Poly(3-nitroanilino)[4-(4-aminophen-1-yldiazo)-1-(3,3-Difluoro-Tetrahydro-4-Hydroxy-5-(Hydroxymethyl)Furan-2-yl)-Pyrimidin-2-one] Phosphazene (9)

I.R.(KBr, cm^{-1}): 1627(N=N), 1635(CON-), 1590(C=N) ,1632(C=C), 1240(P=N), 1135 (C-F), 1306(C-N) , 3470(NH) , 3400(OH).^{1}H-NMR (400MHz, $CDCl_3$, δ, TMS=0, *J*=Hz): 8.2(d, 2 H,Ar-H), 7.71 (d, 2H, Ar-H), 7.1(d, 1H, Pyrimidine, *J*=8.4), 7.8(d, 1H, Ar-H, *J*=2.1), 6.8(d, 1H, Ar-H, *J*=2.1), 6.0-6.1 (m, 3H, Ar-H), 6.2 (d, 1H, Ar-H, *J*=2.7) 6.6(d, 1H, Ar-H, *J*=2.7), 6.4(s, 1H, ribose), 4.6(d, 1H, pyrimidine, *J*=8.4) , 4.4(s, 1H, ribose), 3.9(br, 1H,NH), 3.7(s, 1H, ribose), 3.5(d, 2H, CH_2), 3.2(s, 1H, OH), 2.8(s, 1H, CH).^{31}P- NMR ($CDCl_3$) : δ -18.9 ppm [s ; rel. to $(Ph)_3P$].

Generic steps for the preparation of gemcitabine substituted polyphosphazene-linked azo prodrug (10-13)

The synthetic procedure was the same as above mentioned but 4-[(4-hydroxyphenylazo)-1-(3,3-Difluoro-4-Hydroxy-5-Hydroxymethyltetrahydrofuran-2-yl)]-Pyrimidin-2-one**(3)** (0.250mg,0.0006 moles)in THF(10ml) was used instead of (**2**).

Poly(ethoxy)[4-(4-Hydroxyphen-1-yldiazo)-1-(3,3-Difluoro-Tetrahydro-4-Hydroxy-5-(Hydroxymethyl)Furan-2-yl)-Pyrimidin-2-one] Phosphazene (10)

I.R.(KBr cm^{-1}): 1648(N=N), 1662(CON-), 1575(C=N) ,1616(C=C), 1239(P=N), 1157(C-F), 1326(C-N) , 3410(OH).^{1}H-NMR (400MHz, $CDCl_3$, δ, TMS=0, *J*=Hz): 7.2(d, 1H, pyrimidine, *J*=8.4), 7.6(d, 1H, Ar-H, *J*=2.1), 7.0 (d, 1H, Ar-H, *J*=2.1), 6.5(d, 1H, Ar-H, *J*=2.7) 6.9(d, 1H, Ar-H, *J*=2.7), 6.2(s, 1H, ribose), 4.4(d, 1H, pyrimidine, *J*=8.4) , 4.2(s, 1H, ribose), 4.01(m,2H, CH_2), 3.5(s, 1H, ribose), 3.2(d, 2H, CH_2), 3.0(s, 1H, OH), 2.6(s, 1H, CH),1.29(t,3H,CH_3).^{31}P- NMR ($CDCl_3$) : δ -17.9 ppm [s ; rel. to $(Ph)_3P$].

Poly(t-Butoxy)[4-(4-Hydroxyphen-1-yldiazo)-1-(3,3-Difluoro-Tetrahydro-4-Hydroxy-5-(Hydroxymethyl)Furan-2-yl)-Pyrimidin-2-one]Phosphazene (11)

I.R.(KBr, ν cm^{-1}): 1640(N=N), 1658(CON-), 1565(C=N) ,1620(C=C), 1228(P=N), 1167(C-F), 1326(C-N) , 3395(OH).^{1}H-NMR (400MHz, $CDCl_3$, δ, TMS=0, *J*=Hz): 7.4(d, 1H, pyrimidine, *J*=8.4), 7.8(d, 1H, Ar-H, *J*=2.1), 6.9(d, 1H, Ar-H, *J*=2.1), 6.2(d, 1H, Ar-H, *J*=2.7) 7.0(d, 1H, Ar-H, *J*=2.7), 6.5(s, 1H, ribose), 4.2(d, 1H, pyrimidine, *J*=8.4) , 4.0(s, 1H, ribose), 3.5(s, 1H, ribose), 3.2(d, 2H, CH_2), 3.0(s, 1H, OH), 2.6(s, 1H, CH), 1.19(t, 9H,CH_3).^{31}P- NMR ($CDCl_3$) : δ -18.2 ppm [s ; rel. to $(Ph)_3P$]

Poly(4-Chloroanilino)[4-(4-Hydroxyphen-1-yldiazo)-1-(3,3-Difluoro-Tetrahydro-4-Hydroxy-5-(Hydroxymethyl)Furan-2-yl)-Pyrimidin-2-one] Phosphazene (12)

I.R.(KBr, ν cm^{-1}): 1635(N=N), 1655(CON-), 1570(C=N) ,1660(C=C), 1228(P=N), 1167(C-F), 1326(C-N) , 3395(OH).^{1}H-NMR (400MHz, $CDCl_3$, δ, TMS=0, *J*=Hz): 7.71(d, 2H, Ar-H), 7.49(d, 2H, Ar-H), 7.2(d, 1H,

pyrimidine, *J*=8.4), 7.8(d, 1H, Ar-H, *J*=2.1), 6.9(d, 1H, Ar-H, *J*=2.1), 6.2(d, 1H, Ar-H, *J*=2.7) 7.0(d, 1H, Ar-H, *J*=2.7), 6.5(s, 1H, ribose), 4.2(d, 1H, pyrimidine, *J*=8.4) , 4.0(s, 1H, ribose), 3.5(s, 1H, ribose), 3.2(d, 2H, CH_2), 3.0(s, 1H, OH), 2.6(s, 1H, CH).^{31}P- NMR ($CDCl_3$) : δ -18.2 ppm [s ; rel. to $(Ph)_3P$]

Poly(3-nitroanilino)[4-(4-Hydroxyphen-1-yldiazo)-1-(3,3-Difluoro-Tetrahydro-4-Hydroxy-5-(Hydroxymethyl) Furan-2-yl)-Pyrimidin-2-one] Phosphazene (13)

I.R.(KBr, cm^{-1}): 1630(N=N), 1665(CON-), 1565(C=N) ,1678(C=C), 1215(P=N), 1165(C-F), 1335(C-N) , 3405(OH).^{1}H-NMR (400MHz, $CDCl_3$, δ, TMS=0, *J*=Hz): 8.2(d, 2 H, Ar-H), 7.71 (d, 2H, Ar-H), 7.6(d, 1H, pyrimidine, *J*=8.4), 7.9(d, 1H, Ar-H, *J*=2.1), 7.2 (d, 1H, Ar-H, *J*=2.1), 6.5(d, 1H, Ar-H, *J*=2.7) 7.4(d, 1H, Ar-H, *J*=2.7), 6.7(s, 1H, ribose), 4.5(d, 1H, pyrimidine, *J*=8.4) , 4.2(s, 1H, ribose), 3.8(s, 1H,ribose), 3.4(d, 2H, CH_2), 3.2(s, 1H, OH), 2.9 (s, 1H, CH).^{31}P- NMR ($CDCl_3$) : δ -19.8 ppm [s ; rel. to $(Ph)_3P$]

Synthesis of an azo-based prodrug of gemcitabine (4)

The glacial acetic acid (50 percent v/v) was used to dissolve the p-amino benzoic acid (5 gm.0.0364 moles). A solution of sodium nitrite (4gm, 0.0546moles) in water (16ml) was progressively added to the solution after it had been chilled to 0°C. The reaction mixture's temperature was kept below -5°C. In another flask, phenol (3.42gm,0.0364moles) was dissolved sodium hydroxide (19.74ml). The PABA solution was gradually added to this, and the reaction mixture was agitated for a further two hours. To obtain the desired result, the reaction was allowed to stand before the precipitated product was filtered and dried.

Stirring of Diazotized PABA (2gm,0.0082 moles), Gemcitabine (2.93gm,0.0098 moles), and Dicyclohexylcarbodimide (DCC) (2.018 gm, 0.0098 moles) in THF(40 ml) was done firstly at 0°C for 4 hrs and then continued for 20 hours at room temperature. Further filtration done to remove dicyclohexylurea (DCU). The solvent was evaporated under vaccum to obtain the desired compound (4)

N-(1-((2R, 4S, 5R)-3,3-Difluoro-4-Hydroxy-5-(Hydroxymethyl)Tetrahydrofuran-2-yl)-1,6-dihydropyrimidin-4-yl)-4-((4-Hydroxyphenyl)diazenyl) benzamide (4)

IR (KBr , cm^{-1}) : 3624 (Phenolic OH), 3400(OH), 1644(CON-), 1640(N=N), 1595(C=N), 1140(C-F), 1615(C=C), 1300(C-N), 1670(CONH). ^{1}H-NMR (400MHz, DMSO-d_6, *J*=Hz, δ ppm): 7.27(d,1H, pyrimidine, *J*=8.2), 7.1 (d, 2H, Ar-H, *J*=2.5), 6.9 (d, 4H, Ar-H, *J*=2.8), 6.8 (d, 2H, Ar-H, *J*=2.6), 6.4 (s,1H,ribose H), 4.0(s, (br),1H,OH), 3.8(d, 2H,CH_2), 3.5(s,1H,ribose OH), 3.1 (s,1H, ribose OH).

Experimental procedure: Synthesis of chitosan-drug conjugates (14)

General steps for synthesizing the gemcitabine chitosan-linked azo prodrug (14)

(4) (0.150gm,0.0003moles) was dissolved in acetonitrile (40ml) and triphosgene (0.099gm,0.0001moles) was added, RBF was enclosed with the rubber septum. The stirring took place for 5 minutes. Then, using a syringe, dropwise addition of the chitosan solution (0.049gm, 0.0003moles) in 2 percent acetic acid (15ml) was carried out. Stirring was then carried out for 5 days. The reaction mixture was then filtered, and the extract was then under low pressure concentrated to obtain a brown product. **(14).**

Chitosan linked Prodrug of Gemcitabine

IR (KBr) cm^{-1}: 3624 (Phenolic OH), 3400(OH), 1644(CON-), 1640(N=N), 1595(C=N), 1140(C-F), 1615(C=C), 1300(C-N), 1670(-CONH). ^{1}H NMR (400MHz, DMSO-d_6, *J*=Hz, δ ppm): 7.67(s, I H, N-H) 7.27 (d,1H, Pyrimidine, *J*=8.2), 7.1 (d, 2H, Ar-H, J=2.5), 6.9 (d, 4H, Ar-H, *J*=2.8), 6.8 (d, 2H, Ar-H, *J*=2.6), 6.4 (s,1H, ribose H), 4.0(s, (br),1H,OH), 3.8(d, 2H,CH_2), 3.5(s,1H, ribose OH), 3.1 (s,1H, ribose OH).

2.2 Stability studies

Polymer-drug conjugates (6–14) were tested for stability in simulated alkaline (pH=7.4) and acidic (pH=1.2) conditions. A membrane dialysis bag was filled with the weighed amount (10 mg) of each polymer-linked conjugate and sealed before being placed in a beaker containing 50 ml of 0.1N HCl pH 1.2 and PBS pH 7.4, respectively. The solutions were kept at 370C while being continuously stirred. To measure the drop in polymer-drug conjugate amount in the upper Gastrointestinal medium, a sample (1ml) was removed from the beaker at the appropriate time interval and measured on a UV spectrophotometer at 274nm and 284nm (0.1N HCl, 7.4 pH).

***In-vitro* Release Studies without Caecal Content**

The USP dissolution type II equipment was used for the in-vitro release investigations, and 250.0 ml beakers were inserted in the beaker of the standard USP dissolution apparatus and process was performed at a depth of 40 mm and 100 rpm. Gemcitabine substituted polyphosphazene-linked prodrugs were suspended for 2 hours in simulated gastric fluid (SGF, pH 1.2). The release investigation was then conducted for three hours with simulated intestinal fluid (SIF) with a pH of 7.4. The release investigation was conducted for 19 hours after the dissolving media had been replaced with simulated intestinal fluid with a pH of 6.8. To change the sink condition regularly, 1 ml samples of the dissolution medium were replaced every so often with 1 ml samples, which were then used to analyze the gemcitabine release from the polymer-drug conjugates backbone at maximum concentrations of 274 (pH 1.2) and 284, respectively (pH 6.8 and 7.4).

In-vitro Release Studies with Caecal Content

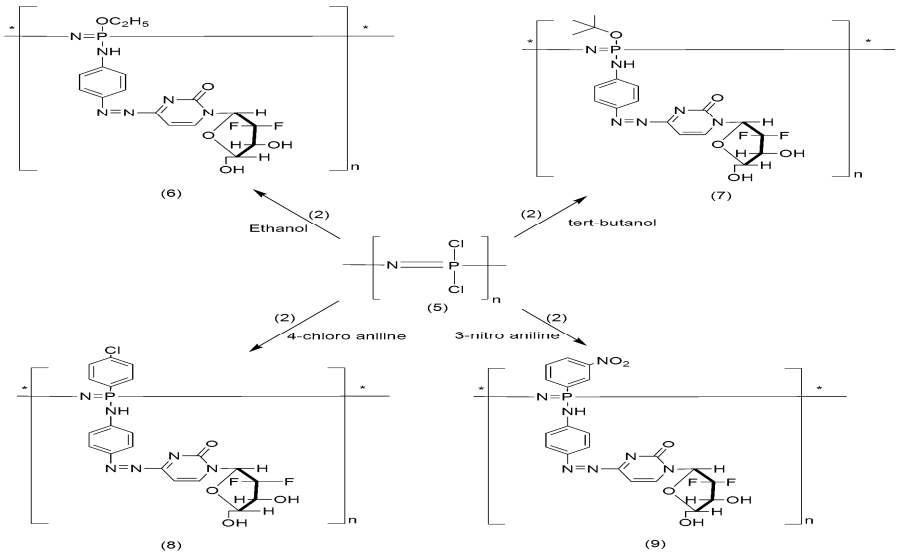
Since rat caecal contents are known to be similar to those of the human intestinal microflora, drug release studies were conducted in their presence to assess the synthesized polymer-linked prodrugs of gemcitabine's sensitivity to biotransformation in the presence of colonic bacteria. For this investigation, Wistar rats weighing 150–200 g were chosen. These were kept up with a regular diet. 10 rats cutted by spinal traction, and the caecum was isolated, tied at both ends, and removed for the removal of caecal content 45 minutes before it was introduced into the dissolution media. Each caecal content sample was transferred to a 10 ml beaker containing PBS 6.8 buffer that had already been weighed (previously bubbled with Nitrogen). The combined content's weight was calculated. To maintain an anaerobic condition, nitrogen was continually circulated through the pooled liquid. With 250.0 ml beakers inserted in the beaker of the standard USP dissolution apparatus, drug release tests were carried out in USP type-II apparatus. The beaker was filled with a measured amount of 187.5 ml of 0.1 N HCl, and the dissolution process was carried out at a depth of 40 mm and 100.0 rpm. The dialysis membrane holding the polymer-drug conjugates was filled with 5 ml of the dissolving media that contained 2% of the rat caecal content. The dialysis bag was tied up at both ends and kept in the USP dissolution apparatus and dissolution was carried out for 2 hrs. PBS pH 7.4 was used as the replacement medium after 2 hours, and dissolution continued for 3 hours. The dissolution process was then carried out for 19 hours with PBS pH 7.4 and samples were taken regularly. To maintain anaerobic conditions, nitrogen was added to the medium at a rate that didn't cause turbulence in the dissolving liquid.

RESULT AND DISCUSSION

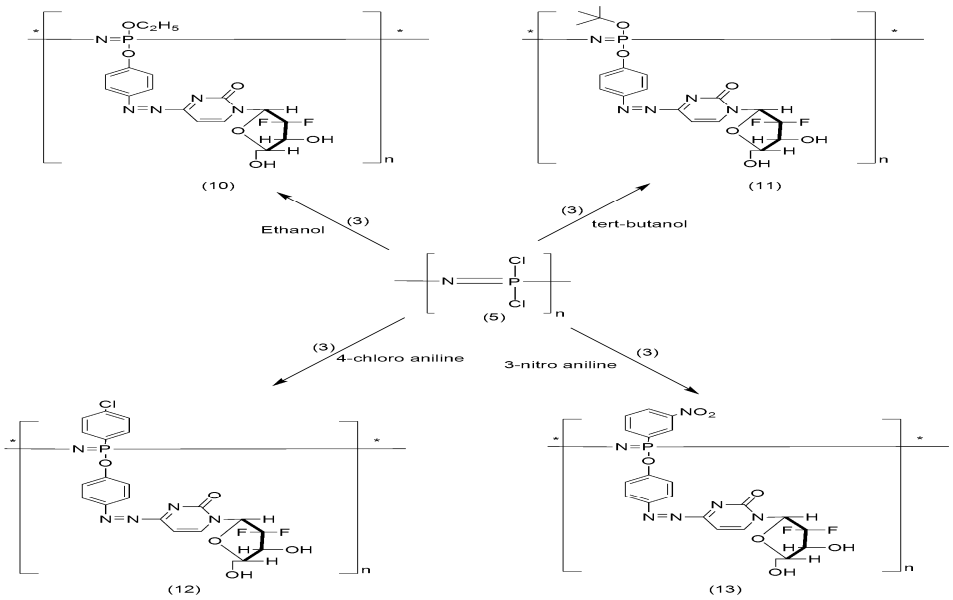
Chemistry

Drug conjugates of chitosan and polyphosphazene were synthesized following schemes 1, 2, and 3. The formula for making gemcitabine prodrug (2,3) was acquired from the literature [Sharma et al., 2013]. The compound (4) was created by diazotizing PABA, coupling it with phenol, and then reacting with gemcitabine and DCC. According to the method described in the literature, low molecular weight polydichlorophosphazene was produced. It wasn't purified in any way before usage. One of the chlorine atoms connected to the polyphosphazene was changed by equimolar substitution with a number of groups, including ethanol, t-butanol, 4-chloroaniline, and 3-nitroaniline, to produce the desired polymer-drug conjugates. The other chlorine atom was replaced with the azo-based prodrugs of gemcitabine (2) and (3). (**6-13**).

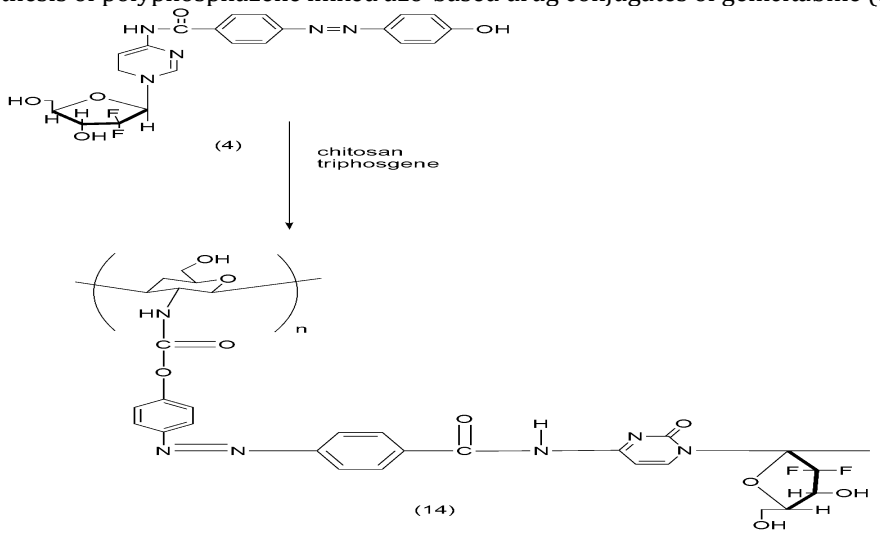
To obtain the desired chitosan-linked derivative, the amino group in chitosan was replaced by the equimolar substitution of (4) by the formation of an amide bond (14). IR and 1H-NMR were used to characterize every compound that was synthesized. The presence of NH, OH, C=N, N=N, and C=C was confirmed by IR spectroscopy features peaks in 3450–3475, 3390–3400, 1590–1600, 1630–1640, and 1618–1628 cm-1 areas. The protons of aromatic regions, pyrimidine, and furan, which are the main key features of conjugation, were observed in 1H-NMR to have characteristic chemical shifts for conjugates (6–14). 31P–NMR observations of phosphorous atom peaks that were close to these values further supported the association of polyphosphazene with drug conjugates.



Scheme 1: Synthesis of polyphosphazene linked azo-based drug conjugates of gemcitabine (**6-9**)



Scheme 2: Synthesis of polyphosphazene linked azo-based drug conjugates of gemcitabine (**10-13**)



Scheme 3: Synthesis of chitosan linked azo-based drug conjugates of gemcitabine (**14**)

Stability studies

Studies on stability were conducted in SGF (pH-1.2) and SIF (pH-7.4) to examine the stability of polymer-drug conjugate. Because of the azoreductase enzyme present in the intestinal microflora, in-vitro stabilityexperiments revealed that the polymer-drug conjugate was stable in SGF and was having its greatest release in SIF. As shown in Figs. 2, 3, and 4, polymer-drug conjugates were stable at intestinal pH as well as in the Upper GIT

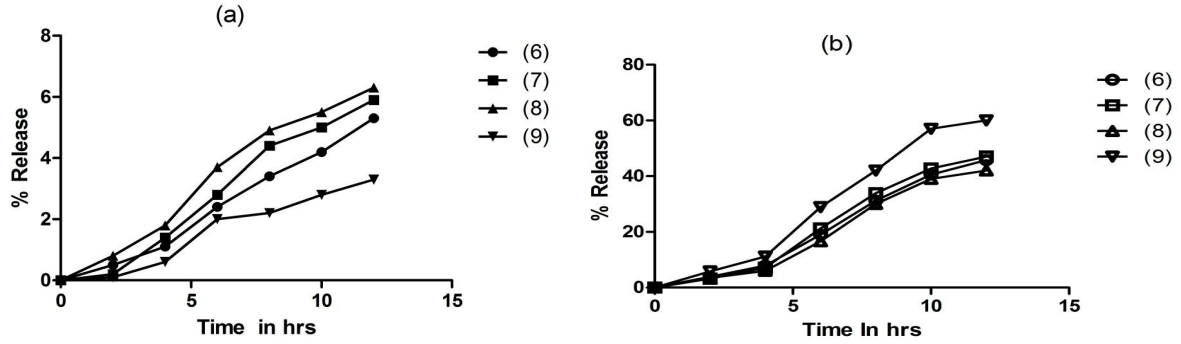


Fig. 2 Stabilities studies of polyphosphazene drug conjugates of Gemcitabine with aniline (**6-9**) in Acidic (pH 1.2) (a) and Alkaline (pH 7.4) (b) buffer at 37°C

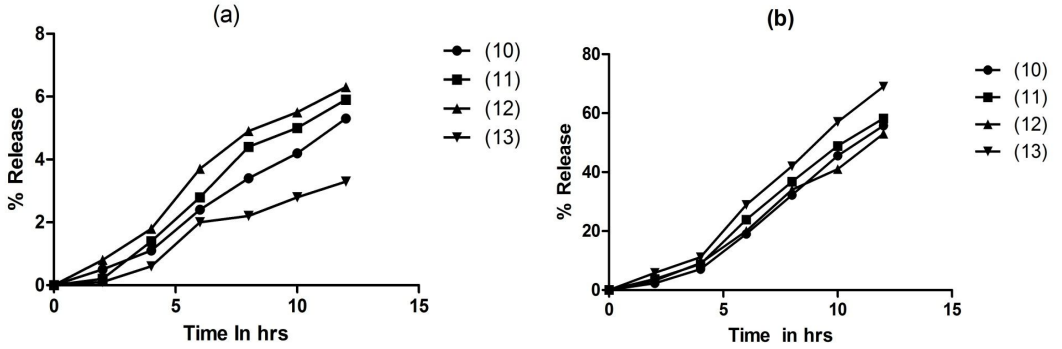


Fig. 3 Stability studies of polyphosphazene drug conjugates of Gemcitabine with phenol (**10-13**) in Acidic (pH -1.2) (a) and Alkaline (pH -7.4) (b) buffer at 37°C.

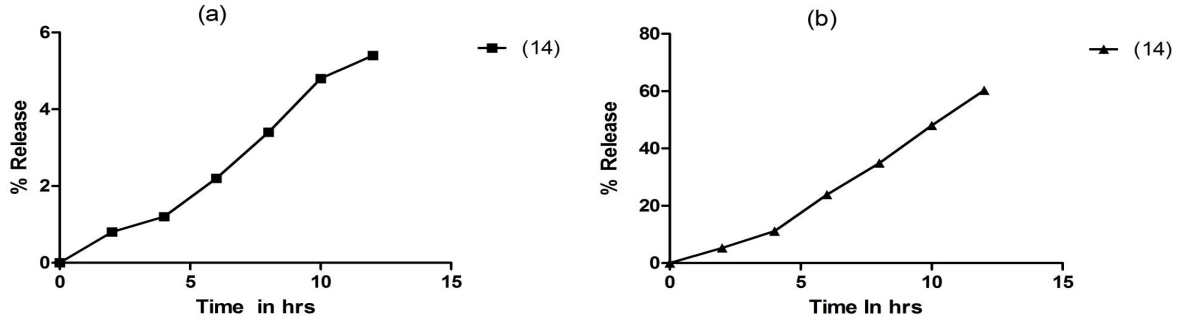


Fig. 4-Stability studies of chitosan drug conjugate of Gemcitabine (**14**) in Acidic (pH-1.2) (a) and Alkaline (pH -7.4) (b) buffer at 37°

***In-vitro* release studies**

In-Vitro release assay was conducted in the absence and presence of rat caecal material to determine the real release behavior of polyphosphazene-linked prodrugs of gemcitabine conjugates to the targeted site, the colon. The polymeric azo bond-based drug conjugate of gemcitabine was analyzed in SGF, or at pH-1.2 for two hours,SIF, or at pH 7.4 for three hours, and finally CPF, or at pH 6.8 for nine hours with and without caecal content. To simulate the conditions from the mouth to colonic transit time, this was done. The following was the order of decreasing polyphosphazene drug conjugate release: In the absence of rat caecal content, as depicted in Figs. 5 and 6, the order of their release pattern was **(13**) (56.2%), **(12)** (55.6%), **(9)** (56.1%), **(12)** (53.6%), **(8)** (53.5%), **(11)** (50.21%), **(6)** (50.45%), **(10)** (47.64%), and **(7)** (47.07%), instead in the presence of rat caecal content, the Pattern of their release was (**13**) (86.87%) > (**12**) (85.16%) > (**9**) (82.47%) > (**8**) (80.8%) > (**11**) (79.55%) > (**7**) (78.5%) > (**10**) (77.63%) > (**6**) (76.03%) as shown in **Fig. 5 and 6.** In the absence of rat caecal content, as depicted in Figure 7, the release pattern of the chitosan-linked drug-conjugate was as follows: (14) 4.0 percent (2h), 14.8 percent (3h), and 49.0 percent (19h), whereas in the presence of rat caecal content, the release pattern was (14) (80.14 percent), as shown in Fig. 7. Comparative investigations between drug conjugates of chitosan and polyphosphazene were carried out (9),(13),(14), as depicted in Fig. 8. All of the drug conjugates demonstrated good release, however compound (13) released gemcitabine to the target site more effectively than the other compounds.

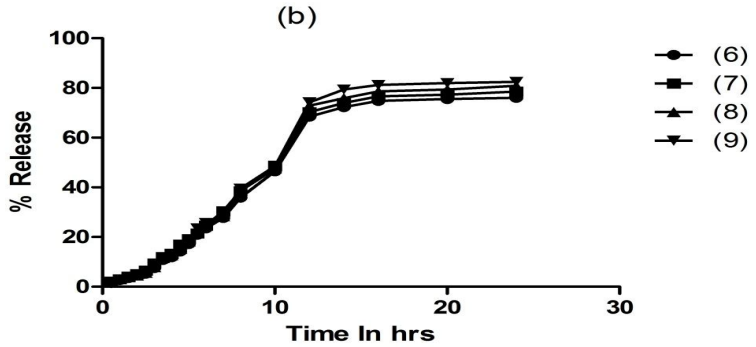


Fig. 5 Cumulative % release of Gemcitabine (**1**) from polyphosphazene linked drug- conjugates with aniline (**6-9**) without rat caecal content (a) and with rat caecal content (b) at 37°C

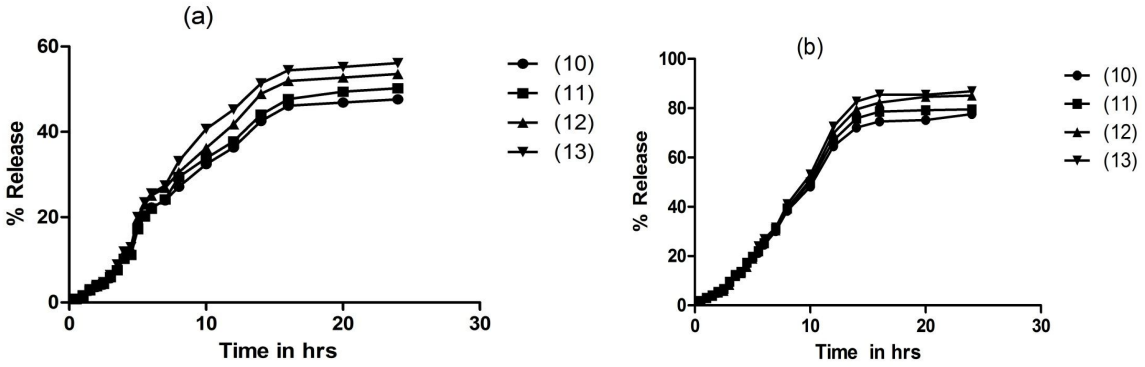


Fig. 6 Cumulative % release of Gemcitabine (**1**) from polyphosphazene drug conjugates with phenol (**10-13**) without rat caecal content (a) and with rat caecal content (b) at 37°C.

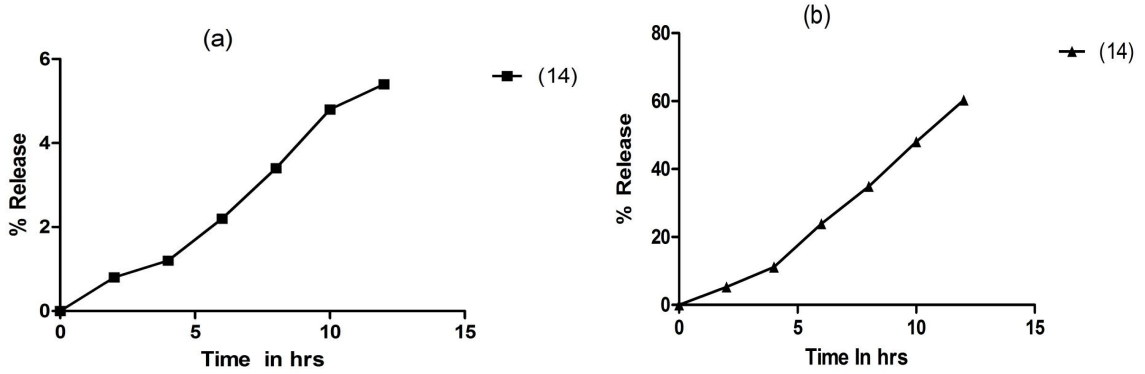


Fig. 7 Cumulative % release of Gemcitabine (**1**) from chitosan drug conjugates (**14**) without Rat Caecal Content (a) and with Rat Caecal Content (b) at 37°C.

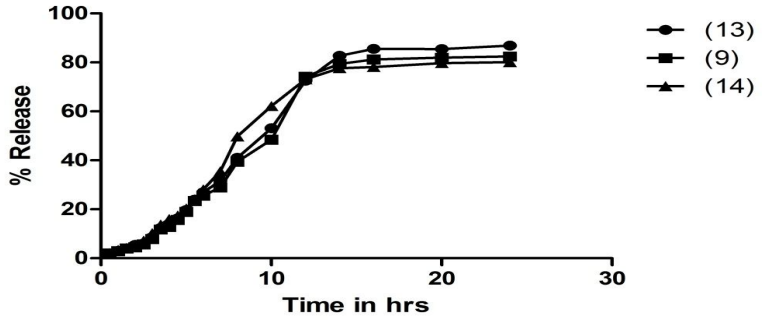


Fig. 8 The comparative cumulative % release of substituted polyphosphazene drug conjugate (**9,13**) and chitosan drug conjugate (**14**). Dissolution media were 0.1 N HCl (2h), pH 7.4 buffer (3h), and pH 6.8 buffer (19h) with rat caecal content.

CONCLUSION

Modern analytical techniques like IR, 1H-NMR and 31P-NMR were used to synthesize and analyze several substituted polyphosphazene and chitosan drug conjugates. Drug conjugates including polyphosphazene and chitosan were tested for physiological in-vitro stability. The findings demonstrated the stability of polymer-drug conjugates in the upper GIT environment. Studies on the release of drugs from polyphosphazene drug conjugates and chitosan drug conjugates with and without rat caecal material were also conducted in vitro. In comparison to the other polymer-drug conjugates, the polyphosphazene drug conjugation (13) demonstrated a maximum release of 86.86 percent, according to the results. When compared to a chitosan-linked azo-based prodrug of gemcitabine (14), it was evident that polyphosphazene-linked azo-based prodrug of gemcitabine (13) exhibited superior drug release (86.86 percent) to the target site, i.e. colon (80.14 percent). To achieve targeted delivery of the anticancer drug gemcitabine to the colon, it might be determined that polyphosphazene-linked prodrugs of gemcitabine may be a superior method to chitosan-linked prodrugs of gemcitabine. As a result, polyphosphazene drug conjugates will be taken into consideration as a possible candidate for future in-vivo release investigations.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest. The research received no specific grant from any funding agency in the public, community, or non-for profit sectors.

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