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INVESTIGATIONS OF ANTIMICROBIAL ACTIVITIES AND DNA INTERACTIONS OF PHOSPHAZENES

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INVESTIGATIONS OF ANTIMICROBIAL ACTIVITIES AND DNA INTERACTIONS OF PHOSPHAZENES

(M.Sc. Thesis)

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ABSTRACT

Phosphazenes have been found to possess antimicrobial activity properties. Additionally, phosphazenes can undergo substitution reactions with different groups. These features have led to an expansion in the field of phosphazene utilization for antibiotic purposes. It has also been found that phosphazenes can bind with DNA, thereby damaging the DNA. This trait is useful when designing drugs that are specifically used to kill cancerous cells. This study focused on investigating the antimicrobial activity and DNA interaction of several phosphazene compounds, numerically labeled as compounds 26, 27, 28, 29, and 30. The microdilution method was used to examine the antimicrobial activity and minimum inhibitory concentration (MIC) values of the phosphazene compounds against 14 different strains of microbes, 11 of which are bacteria and 3 of which are yeast. Additionally, Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) was also measured using the microdilution method. Electrophoresis images were observed after treating the plasmid DNA with the compounds. This was done to see whether or not the compounds restrict DNA. Additionally, to see if the compounds bind with specific nucleotides DNA, the compound-DNA mixture was treated with two different restriction enzymes to determine if the compounds bind to specific nucleotides. The data obtained from control and treatment groups were evaluated and compared. This study found that the MIC values of the compounds used in this study lie in the range of 19.54 to 2500 μM. Among them, compound 30 exhibited the highest potential as an antimicrobial agent against 5 out of the 11 tested bacteria. Also, compound 30 showed strong activity against 2 yeast strains *C. albicans* and *C. tropicalis*. The MIC, MBC, and MFC values of the studied compounds ranged from 19.54 to 2500 μM. All of the studied compounds partly inhibited DNA restriction, indicating that they partly bind to A/A and G/G nucleotides. Some of the compounds used as a result of the research conducted showed antimicrobial activity and also affected DNA.

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FOSFAZENLERİN ANTİMİKROBİYAL AKTİVİTELERİNİN VE DNA ETKİLEŞİMLERİNİN İNCELENMESİ

(Yüksek Lisans Tezi)

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ÖZET

Fosfazenlerin antimikrobiyal aktivite özelliklerine sahip oldukları tespit edilmiştir. Fosfazenler farklı gruplarla yer değiştirme reaksiyonlarına girebilir. Bu özellikleri sayesinde antibiyotik amaçlı kullanılabilme ihtimalleri vardır. Ayrıca, fosfazenlerin DNA ile bağlanarak DNA'ya zarar verebileceği bulunmuştur. Bu özellik, kanser hücrelerini öldürmek için tasarlanmış ilaçlar geliştirilirken faydalı olabilir. Bu çalışmada, 26, 27, 28, 29 ve 30 kodlu fosfazen bileşiklerinin antimikrobiyal aktivitesi ve DNA etkileşimi araştırılmıştır. Fosfazen bileşiklerinin 11'i bakteri ve 3'ü maya olan 14 farklı klinik suşa karşı antimikrobiyal aktivite ve minimal inhibitör konsantrasyonu (MİK) mikrodilüsyon yöntemi ile çalışılmıştır. Ayrıca, minimal bakterisidal/fungisidal konsantrasyon (MBK/MFK) mikrodilüsyon yöntemi kullanılarak ölçülmüştür. Bileşikler pBR322 plazmid DNA'sı ile etkileşime sokularak DNA üzerine etkisi agaroz jel elektroforezi ile gözlemlenmiştir. Bu, bileşiklerin DNA'ya bağlanıp bağlanmadığı ise restriksiyon endonükleaz enzimleri ile kesilerek belirlenmiştir. Bu çalışmada, kullanılan bileşiklerin MİK değerlerinin 19,54 ila 2500 μM aralığında olduğu bulunmuştur. Bunlar arasında, bileşik 30, test edilen 11 bakteriden beş tanesinde bakteriyal büyümeyi inhibisyonu göstermiştir. Ayrıca, bileşik 30'un *C. albicans* ve *C. tropicalis* olmak üzere iki maya suşuna karşı güçlü aktivite gösterdiği tespit edilmiştir. Çalışılan bileşiklerin MİK, MBK ve MFK değerleri 19,54 ila 2500 μM arasında değişmiştir. Çalışılan tüm bileşikler DNA'nın hareketliliğinde zayıf bir etki göstermiştir. Ancak, bileşikler DNA'nın enzim ile kesimini kısmen inhibe etmişdir. Bileşikler DNA'da A/A ve G/G nükleotidlerine kısmen bağlandığı sonucna varılmıştır.

1. Giriş

Siklofosfazenler, iki temel amaç için tarihsel olarak ilgi gören, inorganik halkalı yapıların önemli bir kategorisini oluşturmaktadır. Fosfazen polimerleri, yan gruplardaki değişiklikler aracılığıyla çeşitli fiziksel ve kimyasal özelliklere sahip olmak kapasiteleri nedeniyle son yıllarda önemli ilgi görmüştür. Fosfazenler, güçlü antitümör etkilere sahip olmalarının yanı sıra çeşitli biyomedikal özelliklere ve uygulamalara sahiptir. Araştırmalar, bakteri ve maya hücrelerine karşı biyolojik etkinlikleri üzerine de odaklanmıştır.

Fosfazenler, küresel olarak artan kanser nedeniyle kanserle mücadelede potansiyel birer anti kanser ajan olarak düşünülmektedir. Dünya Sağlık Örgütü'ne göre, kanser dünya çapında ölümlerin önde gelen ikinci nedeni olarak kabul edilmekte olup, 2020 yılında yaklaşık on

milyon ölümle sonuçlanmıştır. Benzer şekilde, ulusal istatistiklere göre, kanserler, 2017 yılında Türkiye'de, kardiyovasküler hastalıklardan sonra ölüm nedenlerinin ikinci sırasında yer almaktadır ve yaşa göre standartlaştırılmış kanser insidans oranları erkeklerde 100 000 kişide 259,2 ve kadınlarda 100 000 kişide 187 olarak belirlenmiştir. Bu önemli ölüm ve insidans rakamları, kanserin önemli bir halk sağlığı sorunu oluşturduğunu göstermektedir. Genellikle, kemoterapi, radyasyon, cerrahi veya bu yaklaşımların bir kombinasyonunu içeren kanser tedavileri oldukça yaygındır.

Fosfazenler önemli antimikrobiyal etkilere sahiptir. Antibiyotikler, son yıllarda insanlarda, tarım, hayvancılık ve su ürünleri yetiştiriciliği gibi çeşitli uygulamalarda bulaşıcı hastalıkların tedavisinde kullanılmıştır. Özellikle sanayileşmiş ülkelerde birçok önemli insan hastalığının etkili bir şekilde kontrol edilmesinde başarılı bir şekilde kullanılmıştır. Ancak, bu başarı artan antibiyotik direnci tehdidi altındadır. Daha önceki yıllarda antibiyotiklere duyarlı olan mikroorganizmaların tedavisi giderek zorlaşmaktadır.

Antibiyotikler, mutasyon, seçilim ve genetik bilginin mikroorganizmalar arasında (yatay gen transferi) akışı yoluyla mikrobiyal genetik ekolojinin tüm yönlerini kuşkusuz etkilemiştir. Bu tez çalışmasında fosfazenlerin klinik mikroorganizmaların üremesine ve DNA üzerine etkileri incelenmiştir.

Ankara Üniversitesi Fen Fakültesi Kimya Bölümü laboratuvarlarında sentezlenen fosfazen bileşiklerinin patojen olan bakteri ve mayalar üzerindeki etkisi Mikrobial Seyreltme (mikrodilüsyon) Yöntemi (MİK) ile araştırılmıştır. Çalışmada kullanılan Gram-negatif bakteri suşları *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 14028 ve *Proteus vulgaris* RSKK 96029'dur. Gram-pozitif bakteri suşları *Bacillus cereus* NRRL B-371, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 ve *Enterococcus hirae* ATCC 9790'dır. Mayalar ise *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258 ve *Candida tropicalis* Y-12968 suşlarıdır. Bakteri ve mayaların antimikrobiyal aktivitesi bileşiklerin minimum inhibe edici konsantrasyon (MİK), minimum bakterisidal konsantrasyon (MBK) ve minimum fungisidal konsantrasyon (MFK) değerleri mikrodilüsyon yöntemi kullanılarak araştırılmıştır. Bileşiklerin plazmid DNA üzerindeki etkileri agaroz jel elektroforezi yöntemi kullanılarak incelenmiştir. Bileşikler DNA'ya bağlanıyorsa, hangi nükleotidlere bağlandıkları belirlemek için bileşiklerle etkileşime girmiş plazmid DNA, *Bam*HI ve *Hind*III restriksiyon enzimleri ile kesilmiştir. Bu araştırma, enfeksiyöz hastalıkların ve kanserin tedavisinde kullanılan ilaçların geliştirilmesine ve halk sağlığı sorunları için önemli sonuçları olan yeni kimyasal ürünlerin sentezine katkıda bulunacaktır.

2. Materyal ve Metot

Materyal

- Fosfazenler

Araştırmada kullanılan orijinal fosfazen bileşikleri, Ankara Üniversitesi Fen Fakültesi Kimya Laboratuvarlarında Prof. Dr. Zeynel KILIÇ ve Prof. Dr. Aytuğ OKUMUŞ ŞAHİN tarafından ve Amasya Üniversitesi Fen Fakültesi Kimya Laboratuvarlarında Dr. Güler İnci Tanrıkulu tarafından sentezlenmiştir. Çalışmada 26, 27, 28, 29 ve 30 olarak adlandırılan beş farklı fosfazen bileşiğinin etkisi incelenmiştir. Bileşikler dimetil sülfoksit (DMSO) içinde

çözülmüş ve 0,45 µm gözenek boyutuna sahip enjektör ucu filtresinden geçirilerek sterilize edilmiştir.

- Kullanılan mikroorganizmalar ve büyüme ortamı

Bu çalışmada, bileşiğin antimikrobiyal aktivitesini belirlemek için Gazi Üniversitesi Fen Fakültesi Moleküler Biyoloji Laboratuvarı kültür koleksiyonundan elde edilen 11 bakteri suşu ve 3 maya suşu kullanılmıştır. Bu bakteriler gram-pozitif ve gram-negatif tiptedir. Gram-negatif bakteri suşları *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 14028 ve *Proteus vulgaris* RSKK 96029'dur. Gram-pozitif bakteri suşları ise *Bacillus cereus* NRRL B-3711, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 ve *Enterococcus hirae* ATCC 9790'dur. Maya suşları ise *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258 ve *Candida tropicalis* Y-12968'dir. Çalışmaya dahil edilen tüm mikroorganizmalar öncelikle stoktan alınmış ve uygun inkübasyon koşullarında aktive edilmiştir. Bakteriler Besiyeri Agar (NA) ortamında, mayalar ise Sabouraud Dekstroz Agar (SDA) ortamında uygun inkübasyon koşullarında aktive edilmiştir.

- Kullanılan kimyasallar

Nutrient Agar (NA) (Merck), Sabouraud Dextrose Agar (SDA) (Merck), Muller Hinton Agar (Merck), Mueller Hinton Broth (Merck), Sabouraud Dextrose Broth (Merck), NaCl (Merck), Dimethyl Sulfoxide (DMSO) (Sigma), Tris (Merck), asetik asit (Merck), etilendiamintetraasetik asit (EDTA) (Merck), agaroz (Applichem), etidyum bromür (Sigma), etanol (Merck), sakkaroz (Sigma), bromofenol mavisi (Merck), Tris-EDTA (TE).

- Kullanılan Tüketim Malzemeleri, Araçlar ve Ekipmanlar

Petri kutuları (90x15 mm) (LP İtaliana), mikropipet uçları (LP İtaliana), şırınga filtre ucu (0,45 µm) (Sartoirus), Erlenmeyer şişeleri, beherler, ölçü kabı gibi çeşitli cam malzemeler, lateks ve nitril eldivenler, halka uçlu öze, pamuk, 96 kuyruklu mikrotitre plakası, çeşitli hacimlerde mikropipetler, otoklav (Tomy SX-500), inkübatör (37°C) ve inkübatör (40°C), kiti saklama dolabı (+4°C) (Sanyo), derin dondurucu (-30 ve -80°C) (Sanyo), jel görüntüleme cihazı (Biometra), güç kaynağı, pH metre (Mettler Toledo), hassas terazi, steril hava kabini, soğutmalı santrifüj (Sigma), yatay elektroforez sistemi, vortex, mikrodalga fırın (Samsung).

- Çözeltiler Hazırlanması

• Fizyolojik tuz çözeltisi
%0,9 oranında NaCl distile su içerisinde çözülerek hazırlanmış ve otoklav kullanılarak 121°C'de 1,5 atm basınçta 15 dakika steril edilmiştir.

• Bakteri ve maya kültür ortamı
Üretici firmanın önerdiği miktarda distile su içerisinde çözülmüş ve steril edilmiş ortam 60°C' ye kadar soğutulduktan sonra katı ortam aseptik koşullarda seril petri kaplarına 18-22 ml dökülmüştür, sıvı ortam ise 10 ml'lik cam tüpler içerisine alınmıştır. Katı ortam katılaştıktan, sıvı ortam da soğuduktan sonra sterilite kontrolü amacıyla 1 gece etüvde

bekletildikten sonra çalışmada kullanılacağı zamana kadar +4°C'lik dolapta muhafaza edilmiştir.

• Sterilizasyon işlemi
Sterilizasyon, otoklav kullanılarak 121°C'de 1,5 atm basınçta 15 dakika yapılmaktadır.

• Tris-asetik asit EDTA (TAE) tamponu (50X)
242 g Tris base, 57,1 ml glacial asetik asit, 100 ml 0,5M EDTA (pH:8), bir miktar distile suda çözüldükten sonra son hacim 1 litreye tamamlanmıştır.

• 0,5M EDTA (pH 8,0)
186,12 g EDTA bir miktar distile suda çözüldükten sonra 800 ml'ye tamamlanmıştır. pH 8 olana kadar NaOH tableti eklenerek karıştırılmıştır. pH ayarlandıktan sonra son hacim distile su ile 1 litreye tamamlanmıştır.

• 1 M Tris-HCl
12,11 g Tris tartılıp bir miktar distile suda çözüldükten sonra konsantre HCl ile pH 8'e ayarlanmıştır. pH ayarlandıktan sonra son hacim distile su ile 100 ml'ye tamamlanmıştır.

• TE (Tris-EDTA) tamponu
1 ml 1 M Tris-HCl (pH 8,0) ve 0,2 ml 0,5 M EDTA (pH 8,0) karıştırılarak son hacim distile su ile 100 ml'ye tamamlanmıştır.

• TAE tamponu
50X TAE tamponundan 20 ml alınıp distile su ile 1 litreye tamamlanmıştır.

• Agaroz jeli
%1 (w/v) agaroz 1X TAE tamponu içerisine alınarak mikrodalga fırın içerisinde kaynatılarak çözülmüş daha sonra belli bir süre soğutulup jel tabağına dökülmüş ve polimerleşmesi beklenmiştir.

• Jel yükleme tamponu
%40 sukroz, %0,025 bromfenol mavisi, %0,25 ksilen siyanol ile hazırlanmıştır.

• Etidyum bromür
10 mg/ml derişimde hazırlanmıştır. Koyu renkli şişelerde muhafaza edilmiştir.

Metot

Antimikrobiyal aktivitenin bileşiklerin belirlenmesi

- McFarland standartına göre mikroorganizma konsantrasyonunun ayarlanması

Bakteriler, tek koloni inokülasyonu yoluyla stok kültürlerden alınan Muller Hinton Agar içeren petri tabaklarına inoküle edilmiş ve 37°C'de 24 saat inkübe edilmiştir. İnkübasyonun ardından aktif olarak büyüyen bakteri kültürleri, McFarland No: 0,5 ($1x10^8$ koloni oluşturan birim (kou)/ml) yoğunluğuna ayarlanmış fizyolojik tuz içeren steril tüplere aktarılmıştır. Maya hücreleri, Sabouraud Dekstroz Agar içeren petri tabaklarına inoküle edilmiş ve 30°C'de 48 saat inkübe edilmiştir. İnkübasyonun ardından aktif olarak büyüyen maya

kültürleri, McFarland No: 0,5 ($1x10^7$ kou/ml) yoğunluğuna ayarlanmış fizyolojik tuz içeren steril tüplere aktarılmıştır.

- Mikrodilüsyon deneyi

Fosfazen bileşiklerinin minimum inhibitör konsantrasyonu (MİK), minimum bakterisidal konsantrasyonu (MBK) ve minimum fungisidal konsantrasyonu (MFK) değerleri mikrodilüsyon yöntemi kullanılarak belirlenmiştir. Sıvı kültür ortamı (bakteriler için Mueller Hinton Broth, mayalar için Sabouraud Dekstroz Broth), her biri 100 µl olmak üzere 96 kuyucuklu bir mikroplakaya dağıtılmıştır. 5000 µM konsantrasyonda bir bileşik alınıp ilk kuyuya 100 µl eklenmiştir. İlk kuyudan aynı miktarda sonraki kuyuya transfer yapılarak bu işlem, 1/2 oranında seyreltilmiş farklı konsantrasyonlara ulaşılıncaya kadar tekrarlanmıştır ve bileşiklerin konsantrasyonlarının 2500 ila 19,54 µM arasında değiştiği gözlemlenmiştir. Pozitif kontrol için, antibakteriyel ajanlar olarak Ampisilin (10 mg/mL) ve Kloramfenikol (30 mg/mL) seçildi ve antifungal ajan olarak Ketoconazole (50 mg/mL) seçilmiştir. McFarland No: 0,5 ($1x10^8$ koloni oluşturan birim (kou)/ml) yoğunluğuna ayarlanmış mikroorganizmalar fizyolojik suda 1:10 oranında seyreltilerek her bir kuyuya 5 µl eklenmiştir. Negatif kontrol olarak sadece kültür ortamı kullanılmıştır. Bakteri içeren mikroplakalar 37°C'de 24 saat, maya içeren plakalar ise 30°C'de 48 saat inkübe edilmiştir. İnkübasyonun ardından, mikroplakadaki her bir kuyudan 10 µl örnek alınmıştır. Bakteriler için örnekler Mueller Hinton agar üzerine ve mayalar için Sabouraud Dekstroz Agar üzerine ekilmiştir. Uygun inkübasyon sürelerinden sonra, farklı konsantrasyonlardaki mikroorganizma çoğalması kontrol edilmiştir. Mikrobiyal büyümenin olmadığı en düşük konsantrasyon, bakteriler için minimum bakterisidal konsantrasyon (MBK) ve mayalar için minimum fungisidal konsantrasyon (MFK) olarak kabul edilmiştir. İnkübasyonun ardından, mikrobiyal büyümenin yavaşladığı ilk konsantrasyon minimum inhibitör konsantrasyonu (MİK) olarak kabul edilmiştir.

- DNA ve bileşiklerin etkileşimi

Fosfazen bileşikleri 26, 27, 28, 29 ve 30'un plazmid DNA üzerindeki etkisi agaroz jel elektroforez yöntemi kullanılarak araştırılmıştır. Bileşiklerin stok çözeltileri dimetil sülfoksitte (DMSO) hazırlanmış ve 1 saat içinde kullanılmıştır. Bileşikler, 5000, 2500, 1250, 625 ve 312,5 µM konsantrasyonlarında plazmid DNA ile 37°C'de inkübe edilmiştir, 24 saatlik inkübasyonun ardından ilaç-DNA karışımı agaroz jel üzerine yüklenmiştir ve oluşan bantlar incelenmiştir. Kontrol olarak, bileşikle etkileşime girmeyen plazmid DNA, jelin ilk kuyusuna yüklenmİştİr.

- Agaroz jel elektroforezi

%1 (ağırlık/hacim) TAE tamponunda agaroz çözeltisi bir jel tepsisine dökülmüş ve polimerizasyona bırakılmıştır. Polimerizasyonun öncesinde jel içinde kuyular oluşturulmuş ve bir ilaç-DNA karışımı ile yükleme tamponu bu kuyulara yüklenmiştir. Elektroforez, TAE tamponunda 70 V'de 3 saat boyunca gerçekleştirilmiştir. Elektroforezin sonunda, jel etidyum bromür ile boyanmıştır. Daha sonra, jeller, ultraviyole ışık altında BioDoc Analyze (Biometra) jel görüntüleme cihazı kullanılarak görüntülenmiş ve görüntüler bir bilgisayarda JPEG formatında kaydedilmiştir.

- *Bam*HI ve *Hind*III restriksiyon enzimi reaksiyonu

*Bam*HI enzimi DNA üzerinde 5'-G/GATCC-3' bölgesini tanırken, *Hind*III enzimi 5'-A/AGCTT-3' bölgesini tanımaktadır. Bileşiklerin bu DNA bölgelerine bağlanmasını anlamak için bileşikler ve DNA 24 saat boyunca inkübe edilmiştir. Daha sonra, bunlar *Bam*HI ve *Hind*III enzimleri ile kesim işlemine tabi tutuldu ve 37°C'de 1 saat daha inkübe edilmiştir. Enzimle işlenmiş ilaç-DNA karışımı, TAE tamponunda %1 agaroz jelinde 70 V'de 1 saat boyunca yürütülmüştür. Elektroforezden sonra, jel etidyum bromür ile boyanmış ve jeller, BioDoc Analyze (Biometra) jel görüntüleme cihazı kullanılarak ultraviyole ışık altında görüntülenmiştir. Görüntüler daha sonra bir bilgisayarda JPEG formatında kaydedilmiştir.

3. Araştırmanın sonuçları

In vitro antimikrobiyal aktivite

26, 27, 28, 29 ve 30 numaralı bileşiklerin 5000 µM konsantrasyonunda seri seyreltme yapılarak antimikrobiyal aktivitesi belirlenmiştir, seyreltmeler 2500, 1250, 625, 312,5, 156,3, 78,1, 39,1 ve 19,54 µM aralığında bileşik konsantrasyonları elde edilmiştir. Karşılaştırma yapabilmek için kullanılan antibiyotikler (ampisilin ve kloramfenikol) ve antifungal ilaçlar (ketokonazol) pozitif kontrol olarak kullanılmıştır.

İnkübasyon süresi sona erdikten sonra, çeşitli mikroorganizmalara karşı bileşiklerin oluşturduğu mikroorganizma büyümeleri gözlemlenmiştir. Bileşikler 26, 27, 28, 29 ve 30 için Minimum İnhibitör Konsantrasyon (MİK) değerleri mikrodilüsyon yöntemi kullanılarak belirlenmiş, antibakteriyel ilaçlar (ampisilin, kloramfenikol) ve antifungal ilaçlar (ketokonazol) pozitif kontroller olarak kullanılmıştır. Ayrıca, Minimum Bakterisidal Konsantrasyon (MBK) ve Minimum Fungisidal Konsantrasyon (MFK) değerleri de mikrodilüsyon yöntemi kullanılarak belirlenmiştir. Bulunan MİK, MBK ve MFK değerleri (<19,54) ila (>2500 µM) arasında değişmektedir.

Bir bileşiğin potansiyeli, MİK değerinin pozitif kontrolün MİK değeriyle karşılaştırılarak değerlendirilmiştir. Bir bileşiğin MİK değeri, pozitif kontrolün MİK değerinden daha düşük bulunursa, o bileşiğin bir inhibitör olarak potansiyeli olduğu düşünülmektedir.

Bileşik 26'nın *E. coli* ATCC 35218'e karşı MİK değeri 1250 µM; *P. aeruginosa* ATCC 27853 ve *K. pneumoniae* ATCC 13883 için 625 µM'dır. Bileşik 26'nın *P. aeruginosa* ATCC 27853 üzerindeki etkisi, *E. coli* ATCC 35218 ve *K. pneumoniae* ATCC 13883 üzerindeki etkisine göre daha yüksektir. Bileşik 27'nin *P. aeruginosa* ATCC 27853 ve *P. vulgaris* RSKK 96029'a karşı MİK değeri 625 µM'dır. Bileşik 27'nin *P. aeruginosa* ATCC 27853 üzerindeki etkisi, *P. vulgaris* RSKK 96029 üzerindeki etkisine göre daha yüksektir. Bileşik 28'in *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029'a karşı MİK değeri 625 µM'dır. Bileşik 28'in *E. coli* ATCC 35218 ve *P. aeruginosa* ATCC 27853 üzerindeki etkisi, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029 üzerindeki etkisine göre daha yüksektir. Bileşik 29'un *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029'a karşı MİK değeri 625 µM'dır. Bileşik 29'un *E. coli* ATCC 35218 ve *P. aeruginosa* ATCC 27853 üzerindeki etkisi, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029 üzerindeki etkisine göre daha yüksektir. Bileşik 30'un *E. coli* ATCC 35218'e karşı MİK değeri 1250 µM; *S. aureus* ATCC 25923'e 78,1 µM; *P. aeruginosa* ATCC 27853, *K.*

pneumoniae ATCC 13883 ve *P. vulgaris* RSKK 96029'a 625 µM; *C. albicans* ATCC 10231'e 156,3 µM ve *C. tropicalis* Y-12968'e <19,54 µM'dır. Bileşik 30'un *P. aeruginosa* ATCC 27853 ve *C. tropicalis* Y-12968 üzerindeki etkisi, *E. coli* ATCC 35218, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 13883, *P. vulgaris* RSKK 96029 ve *C. albicans* ATCC 10231 üzerindeki etkisine göre daha yüksektir.

P. aeruginosa ATCC 27853, tüm bileşiklerin en çok etkilediği mikroorganizmadır. Yedi farklı mikroorganizmaya karşı etkili olan bileşik 30 en etkili bileşiktir.

Bakterisid/fungisid olarak bir bileşiğin potansiyelinin değerlendirilmesi için, MBK/MFK değeri MİK değeri için yapılanlara benzer bir yaklaşımla belirlendi. Bileşiğin potansiyelinin değerlendirilmesi, MBK/MFK değerinin pozitif kontrolün MBK/MFK değeriyle karşılaştırılmasıyla gerçekleştirilmiştir. Pozitif kontrol üç önceden belirlenmiş madde arasındaki en yüksek konsantrasyondan seçilmiştir (ampisilin, kloramfenikol veya ketokonazol).

Bileşik 26'nın *B. cereus* NRRL B-3711, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029'a karşı MBK değeri 1250 µM olarak belirlenmiştir. Bileşik 26'nın *C. tropicalis* Y-12968'e karşı MFK değeri 625 µM olarak belirlenmiştir. Bileşik 26'nın *K. pneumoniae* ATCC 13883, *P. vulgaris* RSKK 96029 ve *C. tropicalis* Y-12968 üzerindeki etkisi eşit derecede yüksek bulunmuştur. Bileşik 27'nin *P. aeruginosa* ATCC 27853 ve *P. vulgaris* RSKK 96029'a karşı MBK değeri 1250 µM olarak belirlenmiştir. Bileşik 27'nin *C. tropicalis* Y-12968'e karşı MFK değeri 625 µM olarak belirlenmiştir. Bileşik 27'nin *P. aeruginosa* ATCC 27853, *P. vulgaris* RSKK 96029 ve *C. tropicalis* Y-12968 üzerindeki etkisi eşit derecede yüksek bulunmuştur. Bileşik 28'in *E. coli* ATCC 25922 üzerindeki MBK değeri 625 µM olarak belirlenmiştir, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029 üzerinde ise 1250 µM olarak belirlenmiştir. Bileşik 28'in *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029 üzerindeki etkisi eşit derecede yüksek bulunmuştur. Bileşik 29'un *E. coli* ATCC 25922 üzerindeki MBK değeri 625 µM olarak belirlenmiştir, *B. cereus* NRRL B-3711'de 312,5 µM olarak belirlenmiştir, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029 üzerinde ise 1250 µM olarak belirlenmiştir. Bileşik 29'un *B. cereus* NRRL B-3711 üzerindeki etkisi, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029 üzerindeki etkisine göre daha yüksek bulunmuştur. Bileşik 30'un *S. aureus* ATCC 25923 üzerindeki MBK değeri 156,3 µM olarak belirlenmiştir, *K. pneumoniae* ATCC 13883'te 1250 µM ve *P. vulgaris* RSKK 96029'da 625 µM olarak belirlenmiştir. Bileşik 30'un *C. tropicalis* Y-12968'e karşı MFK değeri <19,54 µM olarak belirlenmiştir. Bileşik 30'un *C. tropicalis* Y-12968 üzerindeki etkisi, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029 üzerindeki etkisine göre daha yüksek bulunmuştur.

Bu nedenle, bakteri *P. vulgaris* RSKK 96029, tüm bileşikler tarafından en çok etkilenen mikroorganizmadır. Mikroorganizmalar üzerinde en büyük etkiye sahip olan bileşik, beş mikroorganizmaya karşı etkili olması nedeniyle bileşik 29'dur. Pozitif kontrol ve mikroorganizmalar arasında yüksek oranda fark gösteren bileşik 30'dur.

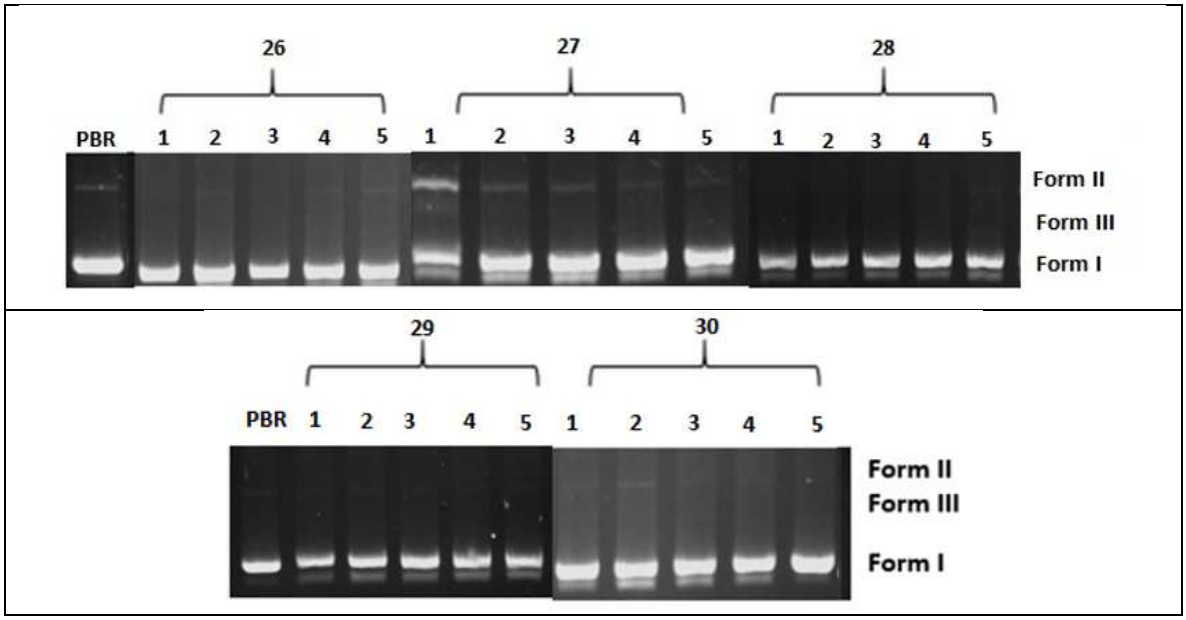
Fosfazen bileşiklerinin DNA ile etkileşimi

DNA ve bileşikler arasındaki etkileşim

Çalışmada kullanılan 26, 27, 28, 29 ve 30 numaralı bileşiklerin plazmid DNA ile etkileşimi agaroz jel elektroforezi kullanılarak araştırılmıştır (şekil 3.1). "PBR" hattı kontrolü temsil eder (etkileşime girmemiş pBR322), ardından gelen ardışık şeritler bileşiklerin azalan konsantrasyonları ile etkileşime girmiş plazmid DNA'yı göstermektedir. Bileşik konsantrasyonlar (1): 5000 µM, (2): 2500 µM, (3): 1250 µM, (4): 625 µM, (5): 312,5 µM. Form I süperheliks plazmid DNA'yı, Form II açık halkasal plazmid DNA'yı, ve Form III doğrusal plazmid DNA'yı temsil etmektedir.

Plazmid DNA'nın DASD substitüanları taşıyan 26 numaralı bileşiğe etkileşimi, bileşiğin konsantrasyonu azaldıkça DNA Form I ve Form II'nin hareketliliğini yavaşlatığı; ayrıca DNA Form I'in yoğunluğunu arttırdığı tespit edilmiştir. Plazmid DNA'nın DASD substitüanları taşıyan 27 numaralı bileşiğe etkileşimi, bileşiğin konsantrasyonu azaldıkça DNA Form I ve Form II'nin hareketliliğini yavaşlatığı; ayrıca DNA Form I'in yoğunluğunu arttırdığı tespit edilmiştir. Ayrıca, Form III'ün varlığı bileşiğin DNA kesimi yaptığını göstermiştir.

Plazmid DNA'nın piperidin substitüanları taşıyan 28 numaralı bileşiğe etkileşimi, bileşiğin konsantrasyonu azaldıkça DNA Form I ve Form II'nin hareketliliğini yavaşlatığı; ayrıca DNA Form II'nin yoğunluğunu arttırdığı tespit edilmiştir. Plazmid DNA'nın piperidin substitüanları taşıyan 29 numaralı bileşiğe etkileşimi, bileşiğin konsantrasyonu azaldıkça DNA Form I ve Form II'nin hareketliliğini yavaşlatığı; ayrıca DNA Form II'nin yoğunluğunu arttırdığı tespit edilmiştir. Plazmid DNA'nın piperidin substitüanları taşıyan 30 numaralı bileşiğe etkileşimi, bileşiğin konsantrasyonu azaldıkça DNA Form I ve Form II'nin hareketliliğini yavaşlatığı; ayrıca DNA Form II'nin yoğunluğunu arttırdığı tespit edilmiştir. Plazmid DNA'nın bileşiğe etkileştiği durumda Form III, gözlenmesi bileşiğin DNA da kırıklara neden olduğunu göstermektedir.



Şekil 3.1. Çalışmada kullanılan bileşiklerin plazmid DNA üzerindeki etkisi

Restriksiyon enzim kesimi

Bileşiklerin DNA'daki nükleotidlere bağlanma afinitesini değerlendirmek için, plazmid DNA, yüksek konsantrasyonlarda (5000 μM) *Bam*HI ve *Hind*III restriksiyon endonükleazları kullanılarak enzimatik kesime tabi tutulmuştur. *Bam*HI enzimi, DNA'daki 5'-G/GATCC-3' bölgesini tanıyıp kesrken, *Hind*III enzimi DNA'daki 5'-A/AGCTT-3' bölgesini kesmektedir. DNA, 26, 27, 28, 29 ve 30 numaralı bileşiklerle etkileşime girdiğinde, *Bam*HI enzimi tarafından kısmen kesilmiştir. Ayrıca, DNA, 26, 27, 28, 29 ve 30 numaralı bileşiklerle etkileşime girdiğinde, *Hind*III enzimi tarafından da kısmen kesilmiştir. Bu, bileşiklerin, nükleotidlerin A/A ve G/G sitelerine bağlanarak DNA ile etkileşime girdiğini göstermektedir.

4. Tartışmalar

Bileşiklerin etkinliği

Çalışılan bileşiklerin, bu çalışmada kullanılan 14 mikroorganizmadan 7'sinin büyümesini kısmen inhibe ettiği bulunmuştur.

MİK oranına dayanarak, *E. coli* ATCC 35218, ampicilline kıyasla bileşikler 26 ve 30'a karşı 2 kat daha duyarlıdır, ancak bileşikler 28 ve 29'a karşı 4 kat daha duyarlıdır. *S. aureus* ATCC 25923, kloramfenikole kıyasla bileşik 30'a karşı 2 kat daha duyarlıdır. *P. aeruginosa* ATCC 27853, test edilen tüm bileşiklere karşı hem ampicilline hem de kloramfenikole kıyasla en az 4 kat daha duyarlıdır. *K. pneumoniae* ATCC 13883, ampicilline kıyasla bileşik 26, 28, 29 ve 30'a karşı 2 kat daha duyarlıdır. *P. vulgaris* RSKK 96029, ampicilline ve kloramfenikole kıyasla bileşik 27, 28, 29 ve 30'a karşı 2 kat daha duyarlıdır. *C. albicans* ATCC 10231, ketoconazole kıyasla bileşik 30'a karşı 2 kat daha duyarlıdır. *C. tropicalis* Y-12968, ketoconazole kıyasla bileşik 30'a karşı en az 4 kat daha duyarlıdır.

MBK/MFK oranına dayanarak, *E. coli* ATCC 25922, kloramfenikole kıyasla bileşikler 28 ve 29'a karşı 2 kat daha duyarlıdır. *B. cereus* NRRL B-3711, ampicilline kıyasla bileşik 26'ya karşı 2 kat daha duyarlıdır, ancak bileşik 29'a karşı 8 kat daha duyarlıdır. *S. aureus* ATCC 25923, kloramfenikole kıyasla bileşik 30'a karşı 16 kat daha duyarlıdır. *P. aeruginosa* ATCC 27853, ampicilline ve kloramfenikole kıyasla bileşikler 27, 28 ve 29'a karşı 2 kat daha duyarlıdır. *K. pneumoniae* ATCC 13883, kloramfenikole kıyasla bileşikler 26, 28, 29 ve 30'a karşı 2 kat daha duyarlıdır. *P. vulgaris* RSKK 96029, ampicilline kıyasla bileşik 26, 27, 28 ve 29'a karşı 2 kat daha duyarlıdır, ancak bileşik 30'a karşı 4 kat daha duyarlıdır. *C. tropicalis* Y-12968, ketoconazole kıyasla bileşikler 26 ve 27'ye karşı 2 kat daha duyarlıdır, ancak bileşik 30'a karşı 64 kat daha duyarlıdır.

Bakteriyel ve fungal duyarlılık

Bu çalışmada kullanılan monospirokiklotrifosfazen bileşiklerinin düzenli olarak 4-dimetilaminobenzil metil pendo koluna sahip olduğu ve diğer pendo kolunun ya 1,4-dioksa-8-azaspiro[4,5]dekan (DASD) ya da pipridin olabileceği tespit edilmiştir. Antimikrobiyal aktivite, 11 patojen bakteri ve 3 patojen maya karşı test edilmiştir. MİK sonuçları, *P. aeruginosa* ATCC 27853 bakterisinin tüm bileşiklerden en çok etkilenen olduğunu ortaya koymuştur. Bu, ya 1,4-dioksa-8-azaspiro[4,5]dekan (DASD) ya da pipridin pendo koluna sahip tamamen substitüe (doldurulmuş) bileşiklerin, *P. aeruginosa* ATCC 27853'ü etkili bir patojen mikroorganizma olarak savaşmada etkili olduğu anlamına gelmiştir. Ek olarak, test

edilen bileşikler *E. coli* ATCC 35218, *K. pneumoniae* ATCC 13883 ve *P. vulgaris* RSKK 96029'u etkilemiş, ancak tüm bileşikler etkilenmemiştir. Genel olarak, MİK sonuçları, bileşikler 26-30'un patojen büyümeyi engellemede etkili olduğunu, ortalama 625 μM civarında bir konsantrasyonda, MBK sonuçlarının ise mikroorganizmayı öldürme etkinliğini gösterdiğini, ortalama 1250 μM civarında bir konsantrasyonda olduğunu göstermektedir. En etkili büyümeyi inhibe eden bileşik 30 (pipridin pendo koluna sahip) olmuştur, 7 farklı mikroorganizma türüne karşı etkili olduğu kanıtlanmıştır (*E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 13883, *P. vulgaris* RSKK 96029, *C. albicans* ATCC 10231 ve *C. tropicalis* Y-12968).

MBK ve MFK sonuçlarına göre bakıldığında, bu çalışmada monospirokiklotrifofazene tarafından en çok etkilenen mikroorganizmaların *P. vulgaris* RSKK 96029 ve *K. pneumoniae* ATCC 13883 olduğu açıktır. Tüm bileşikler çeşitli patojen mikroorganizmalar üzerinde etkiye sahiptir. Ancak, bileşik 30, mikropları öldürme konusunda en etkili bileşiktir, hatta maya olan *C. tropicalis* Y-12968 için pozitif kontrol ketoconazole ile karşılaştırıldığında 64 kat daha fazla hassasiyet göstermektedir.

Mevcut sonuçların gözlemi, piperidin fonksiyonel grubu olan bileşiklerin DASD fonksiyonel grubuna sahip olanlardan daha aktif olduğunu göstermektedir. Özellikle, Gram-negatif bakterilere karşı daha yüksek antimikrobiyal etki göstermektedir. Bu özellik, bileşik 30 ve 27 için geçerlidir. Burada bileşik 30, genel olarak oldukça iyi bir etkinlik gösterirken, bileşik 27'nin sadece 2 bakteri ve mantarlar üzerinde etkili olduğu gözlemlenmiştir.

Bu çalışmanın sonucu, piperidin fonksiyonel grubuna sahip olan bileşiklerin (bileşik 28-30), esas olarak *P. aeruginosa* ATCC 27853 (-), *K. pneumoniae* ATCC 13883 (-), *P. vulgaris* RSKK 96029 (-) ve *C. tropicalis* Y-12968'in neden olduğu enfeksiyonların tedavisi için özel olarak geliştirilebilecek antimikrobiyal ajan olma potansiyeline sahip oldukları desteklemektedir. Bununla birlikte, bu bileşikler *E. coli* ATCC 35218 (-), *E. coli* ATCC 25922 (-), *B. cereus* NRRL B-3711 (-), *S. aureus* ATCC 25923 (+) ve *C. albicans* ATCC 10231'e karşı da etkili olmuştur. Piperidin fonksiyonel grubuna sahip bileşiklerin bakterilere ve mantarlara karşı antimikrobiyal etkisi daha önce halojenobenzen türevi bileşiklerin durumunda rapor edilmiştir. Araştırmaları, piperidinin halojenobenzen türevine nasıl yerleştirildiğinin de antimikrobiyal etkinliği belirlemede kritik olduğunu göstermektedir. Kendisi organik bir yapı olarak, piperidin, tıbbi faydaları olan çeşitli organik bileşiklerin sentezinde bir yapı taşı ve reaktif olarak kullanılmıştır. Piperidin fonksiyonel grubuna sahip geliştirilmiş bir bileşik, bakterilerde yağ asidi sentezini engellediği tespit edilerek bakterileri etkili bir şekilde öldürmüştür. Metal piperidin ditiokarbamat komplekslerinin *S. aureus* ve *C. albicans*'a karşı antimikrobiyal aktivite gösterdiği rapor edilmiştir. Dolayısıyla, bu çalışmalar, mevcut çalışma da dahil olmak üzere, piperidinden türetilmiş bileşiklerin veya piperidin fonksiyonel gruplarına sahip bileşiklerin, yeni bir antibiyotik geliştirilmesi için potansiyel olabileceklerini göstermektedir. İlave çalışmalarla desteklenmesi gerekmektedir.

Kanserle ilgili bileşikler

Kanser, dünya genelinde her altı ölümden birinden sorumlu olan küresel bir sağlık sorunudur. Yavaş yavaş gelişen ve genel bir büyüme kontrolü kaybına yol açan oldukça karmaşık bir dizi hastalık durumudur. Ancak, son dönemdeki gelişmeler kanser gelişiminde yer alan birçok yolun anlaşılmasını ve onlara nasıl hedefleneceğini dramatik bir şekilde iyileştirmiştir. Yeni yaklaşımlar, ilaçlar ve biyolojik moleküller tedavide kullanılmaktadır. Kanser tedavisinde kullanılan birçok ilaç, DNA'yı hedef aldığı için, ilaçlar ile DNA

arasındaki etkileşimler, özellikle antikanser ilaçların geliştirilmesinde, büyük önem kazanmıştır.

Bileşiklerin DNA ile etkileşiminden kaynaklanan DNA konformasyonundaki değişiklikler, DNA'nın DNA nükleobazları ile bağlanma veya kesilme doğasına dayandığı için meydana gelebilmektedir. DNA'nın bileşiklerle etkileşim doğasını değerlendirmek için kullanılan tekniklerden biri plazmid DNA kullanarak yapılan agaroz jel elektroforezidir. Agaroz jel üzerinde gerçekleşen elektroforetik hareketlilik, her bir bileşiğin pBR322'nin süper kıvrılmış konformasyonunun değişmesi, konstrasyonunun değişmesi ve hareketliliği ile belirlenmektedir. Genel olarak, agaroz jel elektroforezi sonuçlarında test bileşikleriyle ekiileşmeyen kontrol plazmidler için (pozitif kontroller) iki temel bant gözlenir. Çok hızlı giden supersarmal Form I DNA, bir iplikte çentik meydan agelmesi sonucu açık halkasal Form II DNA ve iki DNA ipliğinde kesilme sonucu oluşan doğrusal form III DNA. Form I DNA önde jelde yürürken, form II arkada, form II ise iki bant arasında yürür. Bu çalışmada kullanılan fosfazen bileşikleri (26, 27, 28, 29 ve 30) ile plazmid DNA (pBR322) arasındaki etkileşimi incelenmiştir. Tüm bileşikler plazmid DNA da çok az konformasyonel değişikliğe ve hareketinde çok az değişikliğe neden olmaktadır.

Plazmid DNA bileşik ile inkübe edildiğinde, plazmid DNA'nın hareketliliğinde çok az azalma görülmmüştür. Ayrıca, bu çalışmada incelenen bileşiklerin DNA'da kırılma meydana getirdiği ve DNA'da konformasyonel değişikliklere neden olabileceği belirlenmiştir. Bileşiklerle etkileşen tüm DNAlar *Bam*HI enzimi tarafından kısmen ve *Hind*III enzimi tarafından kısmen kesilmiştir. Bu, bileşiklerin DNA ile adenin-adenin (A/A) ve guanin-guanin (G/G) sitelerine bağlanarak etkileşime girdiğini göstermektedir. Bu etkileşim enzimler tarafından tamemen kesilmesini engellemiştir.

*Bam*HI ve *Hind*III, kesim enzimleri olarak, G/GATCC ve A/AGCTT dizilerini tanıyarak işlev görmektedir. Bu kesim enzimleri ayrıca bitişik nükleotid bölgeleri arasındaki fosfodiester bağlarını keser. Bu enzimler ayrıca bileşiklerin G/G veya A/A bölgeleri için afinite belirlemekte kullanılmaktadır. Form I ve Form II'nin Form III'e dönüştürülmesi, bu kesim enzimleri tarafından kesilen DNA'dan kaynaklanmaktadır. *Bam*HI'nin plazmid DNA içinde yalnızca bir bağlanma yeri vardır. Daha önce yapılan araştırmalarda, fosfazen bileşiklerinin DNA ile etkileşimlerinde değişen etkinlik gösterdiği görülmüştür.

Yıllar boyunca, fosfazenlerin biyolojik aktivitesi üzerine yapılan çalışmalar, onların antimikrobiyal ve antikanser ajanlar olarak potansiyellerini ortaya koymuştur. Bu fosfazenlerin biyolojik etkileri, fosfazen bileşiğe bağlı olan fonksiyonel gruplara göre değişmektedir. Bu çalışmada kullanılan fosfazen bileşikleri, DNA ile etkileşime girdiğinde farklı düzeylerde antimikrobiyal ve antikanser aktivite sergilemiştir. Yüksek konsantrasyonlarda (5000 μM), tüm bileşikler yalnızca enzim kesim bölgesindeki nükleotitlerde DNA'ya kısmen bağlanmıştır.

Bakteriyostatik ve bakterisidal ilaç

MİK, bakteriyel büyümenin engellendiği minimum ilaç konsantrasyonunu belirtirken, MBK, bakterilerin öldürüldüğü minimum ilaç konsantrasyonunu belirtmektedir. Genellikle, MBK ile MİK arasında bir ilişki bulunmaktadır.

Bakteriostatik ilaçlar, bakteriyel büyümeyi konak içinde engellemek üzere formüle edilmiştir. Konak içindeki bakterilerin ortadan kaldırılma mekanizması, konak savunma

sistemi tarafından belirlenmektedir. Enfeksiyonun bulunduğu yerde konak savunma mekanizmalarının enfekte edici bakteriyi tamamen yok etmekte yetersiz olduğu durumlarda, bakteriyel büyüme, bakteriler ilacı yok ettikten sonra tekrar başlayabilmektedir. Bu tür senaryolarda, bakterisidal ilaçlar bakterileri tamamen ortadan kaldırmak için gereklidir. Bu iki kategori farmasötik ajan farklı şekilde işlev görür ve MİK ve MBK değerleri, ikisi arasındaki farkı ayırt etmede yardımcı olmaktadır.

Bakteriostatik ilaçlar genellikle MBK değerleri, MİK değerlerinden önemli ölçüde farklı olabilir ve genellikle dört kat daha yüksektir. Bu ilaçların asıl amacının bakterileri ayrım yapmaksızın öldürmektir. Dolayısıyla, ilacın sadece büyümeyi engellemesi ve bakterileri yok etmemesi dezavantaj olarak algılanmamalıdır. Öncelikle bakteriostatik ilaçlara örnek olarak tetrasiklinler, makrolidler ve kloramfenikol verilebilmektedir.

Tablo 4.1, mevcut çalışmada test edilen bileşiklerin farklı bakteri türleri için MİK ve MBK değerleri belirlenmiştir. Bakteriostatik bir ilaç olarak sınıflandırılan kloramfenikol, yeşil renkle gösterilmiştir. Kloramfenikolün, test edilen farklı bakteri türlerine yanıt olarak davranışı, hem MBK hem de MİK'nin bakteri türlerinin değişmesiyle değişebilmektedir. Bakteriostatik bir ilaç için MBK değeri genellikle MİK'nin dört katından daha yüksek olacaktır. Bu durumda, yeşil tonun rengi daha açık olmalıdır. MBK, MİK'nin dört katından daha az olduğunda, yeşil tonun rengi daha koyu olmalıdır. Beklendiği gibi, kloramfenikolün MBK değerleri çoğunlukla MİK'nin dört katından daha fazladır, 11 test edilen bakteriden 7'sinde Bu şekilde gözlenmiştir. Bu gözlem, kloramfenikolün esasen birincil olarak bakteriostatik bir ilaç olduğunu doğrulamaktadır.

4.1 Tablosu'nda ampicillin için koyu bronz renk tonu, onu kloramfenikolden ayırmak için kullanılmıştır. Kloramfenikolün aksine, bir bakteriyosidal ilaç olan ampicilin, MBK'sinin MİK'siyle aynı olması veya en azından dört katı kadar fazla olmamasıyla (daha koyu ten rengi tonu) gösterilmektedir. Test edilen 11 bakteriden, yalnızca ikisinin MBKleri MİK'erinden dört kat daha fazladır (daha açık ten rengi tonu).

4.1 Tablosu'nun analizine dayanarak, bu çalışmada test edilen fosfazen bileşiğinin davranışının kloramfenikolden çok ampicilline benzer olduğu gözlemlenmiştir. Bu ifade, çoğu durumda, bileşik 26-30'un MBK'sinin, MİK'sinin eşit veya dört katından az olduğu şeklinde gösterilmiştir (daha koyu ten rengi tonu). Ancak bazı durumlarda MBK değeri MİK'nin dört katından fazladır (daha açık ten rengi tonu). Bu nedenle, bu çalışmada test edilen fosfazen bileşiklerinin çoğunlukla bakterisidal ilaç adayları olduğu daha çok bakteriostatik ilaçlardan ziyade çıkarılmaktadır.

Çizelge 4.1. Bileşik 26-30'un (bakteriostatik/bakterisidal ilaç) MİK ve MBK değerleri

Compounds							Positive Controls	
Test Microorganisms		26	27	28	29	30	AMP	C
E. coli ATCC 35218 G(-)	MIC	1250	2500	625	625	1250	2500	625
	MBC	2500	2500	2500	2500	2500	2500	>2500
E. coli ATCC 25922 G(-)	MIC	2500	625	312,5	625	2500	<19,5	78,1
	MBC	2500	1250	625	625	2500	<19,5	1250
B. cereus NRRL B-3711 G(-)	MIC	625	>2500	625	312,5	1250	156,3	156,3
	MBC	1250	>2500	>2500	312,5	>2500	2500	1250
B. subtilis ATCC 6633 G(+)	MIC	>2500	1250	>2500	1250	1250	<19,5	78,1
	MBC	>2500	1250	>2500	1250	2500	19,5	78,1
S. aureus ATCC 25923 G(+)	MIC	1250	1250	1250	625	78,1	<19,5	156,3
	MBC	2500	2500	2500	2500	156,3	<19,5	2500
E. faecalis ATCC 29212 G(+)	MIC	625	625	625	625	625	<19,5	312,5
	MBC	>2500	>2500	2500	2500	2500	312,5	625
P. aeruginosa ATCC 27853 G(-)	MIC	625	625	625	625	625	>2500	>2500
	MBC	2500	1250	1250	1250	2500	2500	2500
K. pneumoniae ATCC 13883 G(-)	MIC	625	1250	625	625	625	1250	625
	MBC	1250	2500	1250	1250	1250	1250	2500
S. typhimurium ATCC 14028 G(-)	MIC	625	625	625	625	625	<19,5	156,3
	MBC	2500	2500	2500	2500	2500	<19,5	1250
E. hirae ATCC 9790 G(+)	MIC	625	625	625	312,5	312,5	19,5	156,3
	MBC	2500	>2500	2500	2500	2500	39,1	2500
P. vulgaris RSKK 96029 G(-)	MIC	1250	625	625	625	625	1250	1250
	MBC	1250	1250	1250	1250	625	>2500	2500
Nomenclature:			or		MBC more than 4 times MIC (bacteriostatic drug)			
			or		MBC is 4 times MIC or less (bactericidal drug)			
		↑ Primarily Bacteriocidal		↑ Primarily Bacteriostatic				

5. Sonuç ve Öneriler

- Bu çalışma, monospirokiklotrifosfazen türevleri (bileşik 26-30) üzerine odaklanmıştır. Piperidin ve 1,4-dioksa-8-azaspiro[4,5]dekan (DASD), bileşiklerin astar kollarına yerleştirilen iki fonksiyonel gruptur.

- Bu çalışmada, monospirokiklotrifosfazen bileşiklerinin mikroorganizmalara karşı antimikrobiyal etkinliği, piperidin fonksiyonel grubu bulunanların DASD'ye sahip olanlardan daha etkili olduğunu göstermiştir. Tüm bileşikler, Gram-negatif bakterilere karşı Gram-pozitif olanlardan daha yüksek bir etkinlik sergilemiştir. Belirgin antimikrobiyal etkinliğe sahip bileşiklerin MİK, MBK ve MFK değerleri, mikroorganizmaları düşük konsantrasyonlarda inhibe etme veya öldürme aralığında belirlenmiştir (<19,54) ile (>2500 µM) arasındadır. Bulgular, bu bileşiklerin antimikrobiyal ajanlar olarak potansiyel kullanımını önermektedir ve mikroorganizmalar üzerindeki antimikrobiyal etkilerinin mekanizmalarını aydınlatmak için daha fazla araştırmaya ihtiyaç olduğunu vurgulamaktadır.

- Bileşiklerin DNA ile etkileşimi agaroz jel elektroforezi yöntemi kullanılarak değerlendirilmiştir. Çoğu bileşiğin doğrusal form III DNA oluşturduğu gözlenlenmiştir.

Ayrıca, bileşiklerin DNA ile etkileşime girdiği ve hareketliliğini azalttığı bulunmuştur. Bu, bileşiklerin DNA'ya kovalent olmayan elektrostatik etkileşimler aracılığıyla bağlanabileceğini ifade etmektedir. *Bam*HI ve *Hind*III ile enzimatik kesim, bazı bileşiklerin DNA'nın belirli bölgelerine bağlandığını ortaya koymuştur. DNAdaki diğer bölgelere bileşiklerin bağlanıp bağlanmadığını bulmak için C/C ve T/T'yi kesen restriksiyon enzimleri ile kesim de önerilmektedir.

- Bu tezde incelenen tüm biyolojik aktivite deneylerinin sonuçlarına göre, piperidin substitüentler içeren bileşiklerin daha yüksek aktivite sergilediği açıktır.

Özetle, araştırma, piperidin yan gruplarına sahip fosfazen türevlerinin etkili antimikrobiyal ve antikanser ajanlar olarak hizmet etme potansiyeline sahip olduğunu öne sürmektedir. Fosfazen bileşiklerinde diğer biyolojik aktivitelerin incelenmesi ve dikkate alınması, bu alandaki yeni araştırmalara katkı sağlayabilmektedir.

Bilim Kodu : 20326
Anahtar Kelimeler : Antimikrobiyal aktivite, DNA etkileşimleri, Fosfazenler
Sayfa Adedi : 64
Danışman : Prof. Dr. Leyla AÇIK

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SYMBOLS AND ABBREVIATIONS

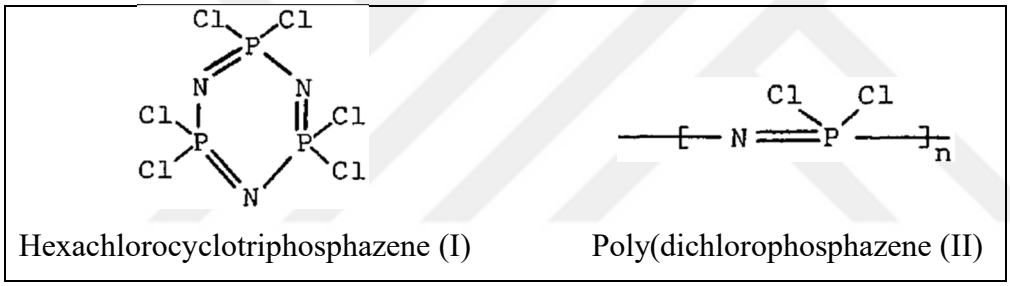
The symbols and abbreviations used in this study are presented below, along with their explanations.

Symbols	Explanations
°C	Degrees Celsius
ml	Millilitre
µl	Microlitre
µM	Micromolar
µm	Micrometer
mm	Milimeter
V	Volt
%	Percent

Abbreviations	Explanations
ATCC	American Type Culture Collection
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
GC	Guanine-Cytosine
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
NRRL	Northern Regional Research Laboratory
RNA	Ribonucleic Acid

1. INTRODUCTION

Phosphazenes, also known as phosphonitrilic compounds, are a group of cyclic and polymeric molecules characterized by phosphorus and nitrogen atoms that are interconnected alternatingly within their structure. The exploration of phosphazenes traces back to 1834 in Figure 1.1, when Liebig and Wohler first documented the synthesis of hexachlorocyclotriphosphazene (I) from phosphorus pentachloride and ammonia. A significant breakthrough occurred in 1897 when Stokes discovered that the cyclic trimer C D could undergo thermal polymerization, resulting in the formation of what was then termed "inorganic rubber," now recognized as poly(dichlorophosphazene) (II). These pioneering investigations laid the groundwork for the emergence of the field of phosphazene chemistry (Allen, 1976).



Hexachlorocyclotriphosphazene (I) Poly(dichlorophosphazene (II)

Figure 1.1. Synthesis of compounds (Allen, 1976)

Cyclophosphazenes constitute a significant category of inorganic ring structures that have historically garnered interest for two primary purposes: generating phosphazene derivatives at the small-molecule level and synthesizing polymeric phosphazene derivatives (Koran et al., 2013). Phosphazene polymers have gained significant interest lately because of their capacity to be customized for a diverse physical and chemical properties through alterations in the side groups (Aslan et al., 2008). Phosphazenes exhibit various biomedical properties and applications, such as potent antitumor effects (Jun et al., 2005). Research has also investigated the efficacy of various phosphazane derivatives in inhibiting and/or killing bacteria and yeast cells (Yilmaz et al., 2002), which shows phosphazanes antimicrobial effects. Polyphosphazene compounds can be utilized as anticancer agents, chemotherapy agents, dyes, fertilizers, and catalysts through the polyphosphazene compound model. This is because polyphosphazene compounds are capable of producing cyclolinear or cyclomatrix substrates as the basic material for biological products (Gleria and De Jaeger, 2001).

Cyclotriphosphazenes are a group of inorganic heterocyclic rings that include phosphorus-nitrogen double bonds (İbişoğlu et al., 2017). These molecules contain reactive phosphorus-halogen bonds, such as chlorine atoms, which readily undergo substitution reactions with various organic groups. By substituting these bonds, new derivatives with diverse properties can be synthesized, depending on the nature of the substituent groups (Uslu et al., 2017). For instance, they have been widely utilized as both anticancer and antimicrobial agents (Şeker et al., 2018).

Phosphazenes have gained considerable significance as anticancer agents due to the escalating global concern over cancer, which now has been reported to be one of the most prominent causes of death all over the world (Ozsoy and Ozay, 2023; Siegel et al., 2023). According to the World Health Organization, cancer ranks as the second most common cause of death, resulting in approximately ten million fatalities worldwide in 2020 (WHO, n.d.). Likewise, according to national statistics, cancers rank as the second leading cause of death after cardiovascular diseases, with age-standardized cancer incidence rates of 259.2 and 187 per 100 000 in males and females, respectively, in Turkey in 2017. These substantial mortality and incidence figures indicate that cancer poses a significant public health challenge (Yuce et al., 2023). Generally, cancer treatments, which often involve chemotherapy, radiation, surgery, or a combination of these approaches, are highly prevalent (Ozsoy and Ozay, 2023).

The development of new compounds, including phosphazene derivatives, as antimicrobials is crucial as the resistance of microbes againts antibiotics was found in many studies as a recent development (Abushaheen et al., 2020). Antibiotics have found a broad use case, from treatment of infectious diseases in humans to various other applications such as agriculture and animal care. This period marked a remarkably successful era in the history of medicine, during which many diseases that had major implication to the population were effectively controlled, particularly in industrialized nations. However, this achievement is now jeopardized by the escalating prevalence of antibiotic resistance. Microorganisms that were once susceptible to antibiotics are increasingly challenging to treat (Mazel and Davies, 1999).

The impact of antibiotics on microbial genetic ecology cannot be overstated. It has influenced all aspects through horizontal gene transfer between microbes, which includes

processes like selection, mutation, and any means of gene transfer. A large, uncontrolled microbiological experiment occured in the second half of the 20th century. This research aims are to complement some existing studies, as their number still needs to be higher on a global research level. This study analyzed, the effects of phosphazenes on antimicrobial activity in microorganisms and their interaction with plasmid DNA. The classification of anticancer drugs can be done according to their mechanisms in which they become effective, e.g., DNA binding, and DNA cleavage.

The focus of this study is to gain insight of the activity of several phosphazene compounds synthesized in the laboratories of the Chemistry Department, Faculty of Science, Ankara University, tested against human pathogens such as bacteria and yeasts. The bacteria and yeast by which antimicrobial activity of the phosphazane compounds were tested are listed in Table 1.1. The bacteria consist of a mix of both gram positive and negative. Measurement of the activity were done by recording both Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of the compounds using microdilution method. The effects of the compounds on plasmid DNA were investigated using agarose gel electrophoresis. In the case where the compounds bind to DNA, to which nucleotides they bind to be determined. The treated plasmid DNA was cleavaged with *Bam*HI and *Hind*III restriction enzymes. This research will contribute to developing drugs used to treat of infectious diseases and cancer, which have significant implications for public health issues, as well as the synthesis of new chemical products.

Table 1.1. Bacteria and yeast that were tested against

Bacteria		
Gram (+)/(-)	Species	Strain Number
(-)	*Escherichia coli*	ATCC 35218, 25922
	Pseudomonas aeruginosa	ATCC 27853
	Klebsiella pneumoniae	ATCC 13883
	Salmonella typhimurium	ATCC 14028
	Proteus vulgaris	RSKK 96029
(+)	*Bacillus cereus*	NRRL B-371
	Bacillus subtilis	ATCC 6633
	Staphylococcus aureus	ATCC 25923
	Enterococcus faecalis	ATCC 29212
	Enterococcus hirae	ATCC 9790

Table 1.1. Bacteria and yeast that were tested against (continued)

Yeast		
	Species	Strain Number
N/A	*Candida albicans*	ATCC 10231
	Candida krusei	ATCC 6258
	Candida tropicalis	NRRL Y-12968

2. THEORETICAL FOUNDATIONS AND LITERATURE REVIEW

2.1. Phosphazenes and Their General Characteristics

Phosphazane compounds are defined by their composition, containing both nitrogen and phosphorus atoms. They feature alternating single and double bonds their molecular backbone's nitrogen and phosphorus atoms. These phosphazanes exhibit diverse structural variations, as illustrated in Figure 2.1, where the arms represented by "R" signify additional groups attached to the molecular backbone (Singler et al., 1984). What sets phosphazanes apart is their unique inorganic nature, in contrast to the predominantly organic composition of most polymers, which are based on carbon. Polyphosphazenes encompass various categories of organic-inorganic polymers, demonstrating the adaptability of the "R" groups, which can be organic or inorganic. Despite limited backbone options, the potential for substituting these "R" groups offers a multitude of possibilities for altering the properties of the resulting compounds, leading to a wide range of materials with distinct characteristics (Allcock, 1972).

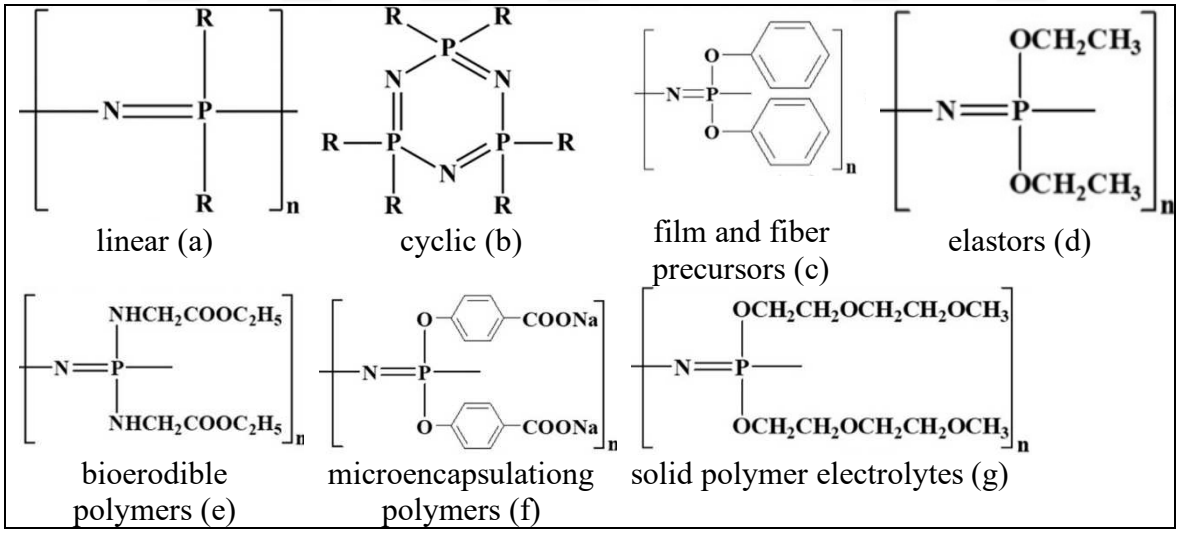


Figure 2.1. Form of polyphosphazenes (Ahmad et al., 2022)

The history of phosphazene dates back to 1834 when Wohler and Rose conducted experiments involving NH_3 and PCl_5. Remarkably, their reaction yielded a pure white crystal compound as a product, without any decomposition (De Jaeger and Gleria, 1998). This discovery laid the foundation for further research, including the work of Gladstone and Holmes. They conducted experiments using the vapor density method, ultimately

formulating the compound's name as $(NPCl_2)^3$ (Allcock, 1968). The name differed from the earlier formulation, $NPCl_2$ in 1844. These historical developments marked significant milestones in understanding of phosphazene compounds and their nomenclature.

Stokes pioneered the study of polyphosphazene compounds by working with cyclic structured forms and describing them as inorganic rubber (Allcock, 2006). His research involved heating chlorophosphazenes to produce these materials. However, progress in the field slowed down after Stokes' work until a significant turning point came with the experiments conducted by Allcock, Valan, and Kugel. Their studies revitalized the research area of phosphazenes and laid the foundation for the field we now recognize (Allcock and Kugel, 1969). Their research yielded several key findings: (i) They demonstrated that certain P-Cl bonds exhibit excellent reactivity, allowing the formation of organic and inorganic hybrid polymers; (ii) They successfully controlled parameters such as temperature, time, reaction termination, and purity to produce hexachlorocyclophosphazene (HCCP) from Poly(dichlorophosphazene) (PDCP). This process involved converting a cyclic trimer into a linear polymer; (iii) By subjecting PDCP to a nucleophilic substitution reaction using alkali metal salts of alcohols and primary or secondary amines, they could create compounds that were resistant to hydrolysis. Furthermore, Allcock proposed the IUPAC name for $[NPCl_2]^3$ as 2,2,4,4,6,6-hexachloro-1,3,5,2,4,6-triazotriphosphorine, which is now commonly known as HCCP (Allcock, 1972). This work greatly benefited the synthesis of polyphosphazenes, serving as the foundation for developing unique phosphazene compounds with distinctive properties (Ahmad et al., 2022).

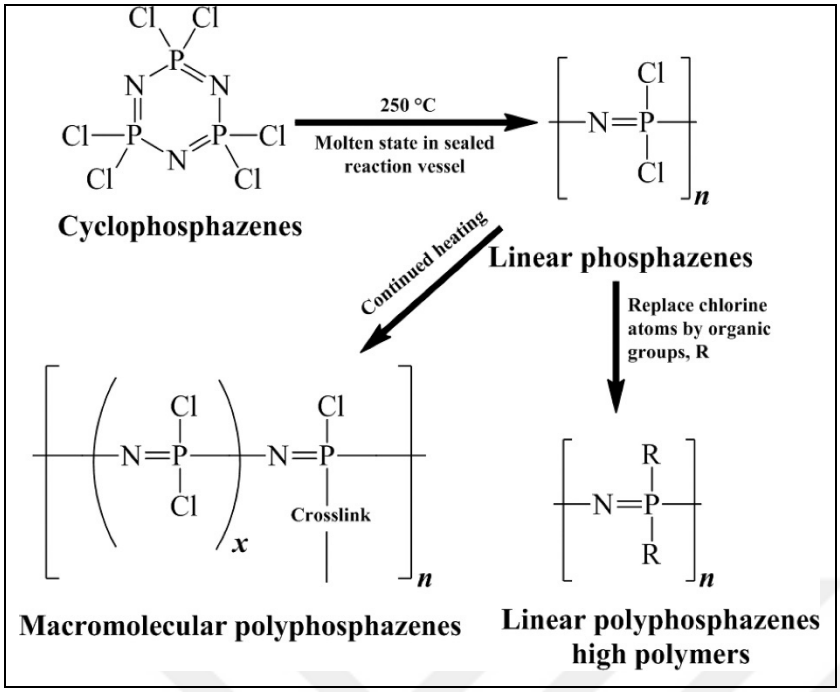


Figure 2.2. Polyphosphazanes and some of the diversity regarding their structure (Ahmad et al., 2022)

2.2. Infectious Diseases and Their Agents

Microorganisms, often referred to as microbes, encompass a wide array of tiny living entities that are not visible to the naked eye. They can include bacteria, fungi, protozoa, and even viruses, with the classification of viruses as microorganisms sometimes debated (NCI, n.d.). Microbes engage in various interactions with their hosts, and one such relationship is mutualistic symbiosis, defined by a benign coexistence that benefits both microbes and hosts (Hooper, 2009). However, microorganisms can also exert adverse effects, causing diseases. The extent to which an organism can induce a disease is quantified as its pathogenicity. The human body employs defense mechanisms to combat pathogenic bacteria, although these bacteria can evade the immune system and utilize the body's resources to cause diseases. In the case of viruses, their virulence is determined by their invasiveness and ability to produce toxins. The progression of a disease following bacterial or viral transmission depends on host factors, including genetic makeup, nutritional status, age, duration of exposure to the organism, and any coexisting illnesses in the host. Environmental factors, such as air pollution and chemical contaminants, also play a role in influencing the body's immune response to bacterial infections (Doron and Gorbach, 2008).

The human microbiota refers to the diverse collection of microorganisms that inhabit specific locations within the body. The types of microorganisms in various parts of the body are shown in Table 2.1. These microorganisms, including bacteria, viruses, and fungi, are crucial in supporting the body's immune system in its fight against infectious diseases. One key mechanism by which they aid in this defense is acting as a protective barrier against invading pathogens. However, pathogens are not easily thwarted, as they have evolved sophisticated strategies to compete with the indigenous microbes within the body. When the balance of these microbial communities is disrupted, a condition known as dysbiosis may occur, potentially worsening diseases. Dysbiosis adversely affects the protective barrier formed by the microbiota, making it less effective in preventing infections. Furthermore, dysbiotic microbiota can employ different mechanisms, including influencing the transfer of virulence factors and creating antibiotic-resistance genes within invading pathogens, thereby enhancing their potency (Davis, 1996).

Table 2.1. Microorganisms found in the normal flora of humans (Davis, 1996)

Anatomical Region	Genus
Skin	*Staphylococcus, Micrococcus, Corynebacterium, Propionibacterium, Streptococcus, Enterobacter, Klebsiella, Escherichia, Proteus*
Oral	*Streptococcus, Staphylococcus, Neisseriae, Diphtheroids* and others
Gastrointestinal	*Helicobacter, Streptococcus, Lactobacillus, Bacteroides*
Urogenital	*Lactobacillus, Staphylococcus, Streptococcus, Escherichia, Corynebacteria, Torulopsis* and *Candida*
Conjunctival	*Corynebacteria, Neisseriae, Moraxellae and Haemophilus*

The gravity of infectious diseases cannot be underestimated, given that they have now become the second leading cause of death worldwide. This issue extends beyond the borders of individual countries; it is both a domestic public health concern and a pressing international challenge. Infectious diseases are consistently factored into policy considerations for the 21st century due to their far-reaching implications. The underlying problem lies in the constant evolution, emergence, and reemergence of these diseases. These ongoing changes have a profound global impact, shaping the role that infectious diseases play in this century and highlighting the critical need for effective strategies to combat them (Fauci, 2001).

There are four primary groups in the realm of infectious agents such as bacteria, viruses, fungi, and parasites. This study, however, directs its attention specifically towards exploring the antimicrobial properties of phosphazenes concerning several bacteria and fungi responsible for causing diseases. The research aims to shed light on which specific compounds under investigation can disrupt the replication of these agents within the host's body or inhibit their production of harmful toxins, offering valuable insights into potential therapeutic interventions.

2.2.1. Bacteria

Bacteria are the most abundant and simplest prokaryotic microorganisms in the world. Prokaryotes lack a nucleus and complex organelles. Since most prokaryotes are smaller than ten micrometers (μm) (Mohamad et al., 2014).

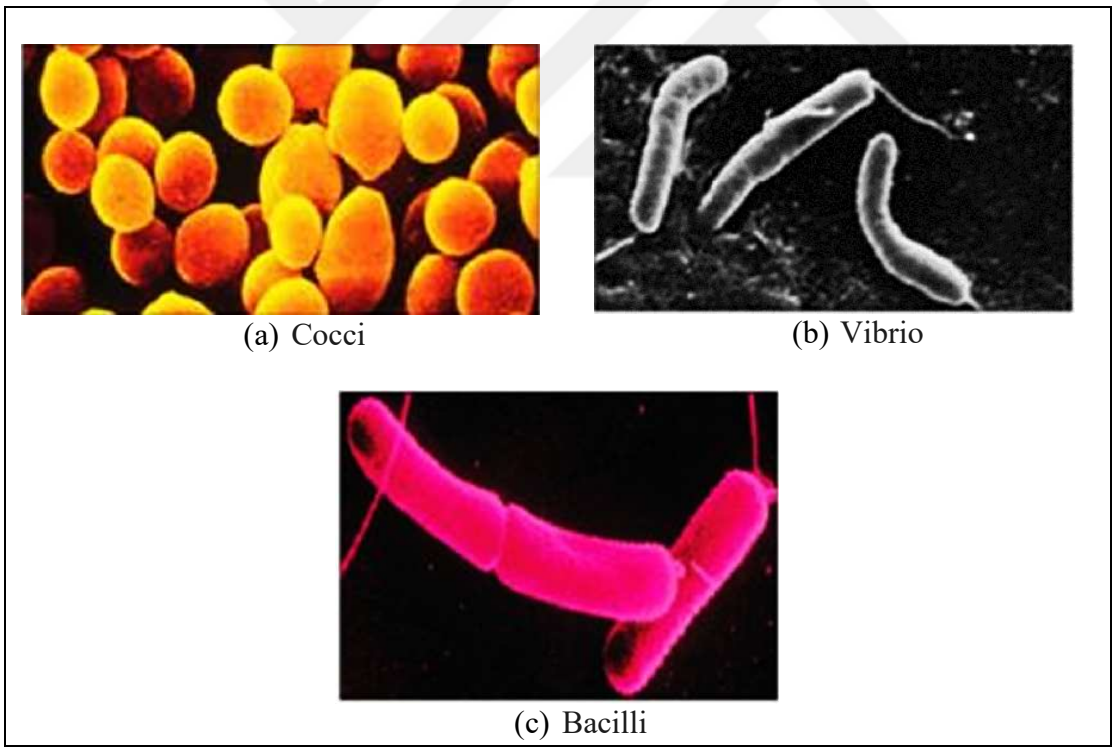


Figure 2.3. Three classes of bacteria (Akbaş et al., 2016)

Prokaryotic organisms are defined by their lack of a cell nucleus and other membrane-bound organelles (Kerfeld et al., 2005). The term "prokaryote" itself signifies "before the nucleus," signifying that the chromosomal DNA in these organisms is not enclosed within any membrane but instead resides freely in the cell cytoplasm. This starkly contrasts eukaryotic

organisms, where the nucleus encapsulates the genomic DNA within a membrane-bound structure. In prokaryotes, the genomic DNA is concentrated within a region called the nucleoid, but it remains unenclosed by membranes (Fuerst, 2010; Stanier and Van Niel, 1962).

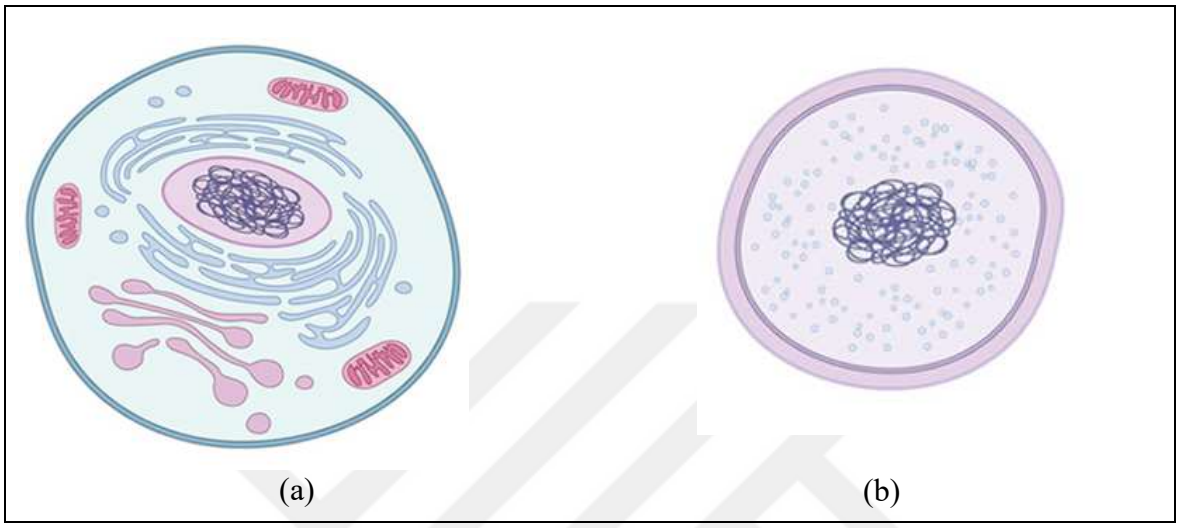


Figure 2.4. Comparing basic eukaryotic and prokaryotic differences (Mäkelä et al., 2011)

Transcription is the process of converting DNA into RNA, while translation involves synthesizing proteins based on the information encoded in RNA. In prokaryotes, which include bacteria, transcription and translation are often coupled, allowing for rapid protein synthesis. This coupling can initiate during the early stages of transcription elongation (Mäkelä et al., 2011). Prokaryotes differ from eukaryotes in the organization of their genetic material. Eukaryotes package their DNA around histone proteins, creating a compacted chromatin structure within the nucleus. In contrast, prokaryotes generally lack histones (except for species in the Archaea), but use supercoiling to condense their DNA into a smaller space (Griswold, 2008). Additionally, prokaryotic cells are protected by a cell membran , which, though structurally simple (Silhavy et al., 2010), serves as a vital barrier safeguarding the cell's contents.

The environments where bacteria thrive can be inherently hostile and fraught with unpredictability. To navigate these challenges, bacteria have developed a complex multilayered structure known as the cell envelope. This envelope can be broadly categorized into two distinct types: gram-negative and gram-positive. Gram-negative bacteria can be differentiated by their cell wall that consist of thin peptidoglycan, followed by an outer

membrane composed of lipopolysaccharides. A well-known example of a gram-negative bacterium is *E. coli*, as depicted in Figure 2.5. The term "peptidoglycan" hints at its composition, involving both "glycan" and "peptide" components. What makes up for Glycans are N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc), represented as gray and blue hexagons, respectively, in Figure 2.5. In contrast, peptide chains are composed of l-alanine, d-glutamic acid, meso-diaminopimelic acid, and d-alanine, presented as l-ala, d-glu2, mDAP, and d-ala in Figure 2.5 (Garde et al., 2021). Gram-positive bacteria are missing an outer most membrane. However, it is surrounded by peptidoglycan layer that are usually much thicker than gram-negative bacteria (Silhavy et al., 2010). The differences between gram-positive and gram-negative bacteria, including their outer structure, can be found in Figure 2.6.

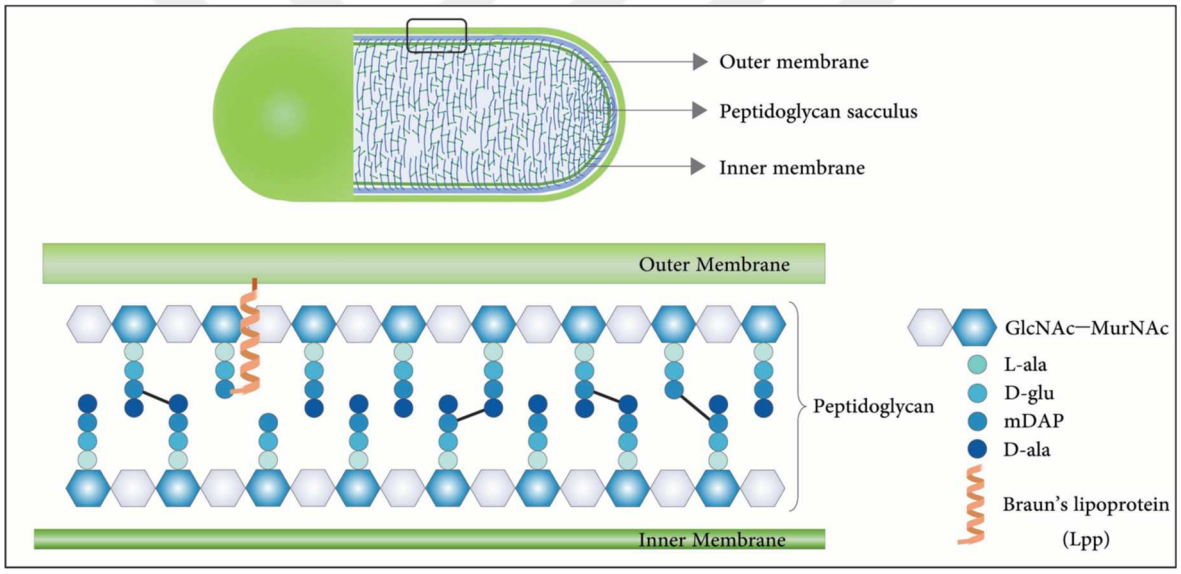


Figure 2.5. Chemical composition and structure of peptididoglycans. (Head) Diagram of a rod-shaped *E. Coli* cell, showing the location of the peptidoglycan sacculus (blue mesh) between the OM and IM. (Base) The peptidoglycan's composition is seen in the magnified rectangle (Garde et al., 2021)

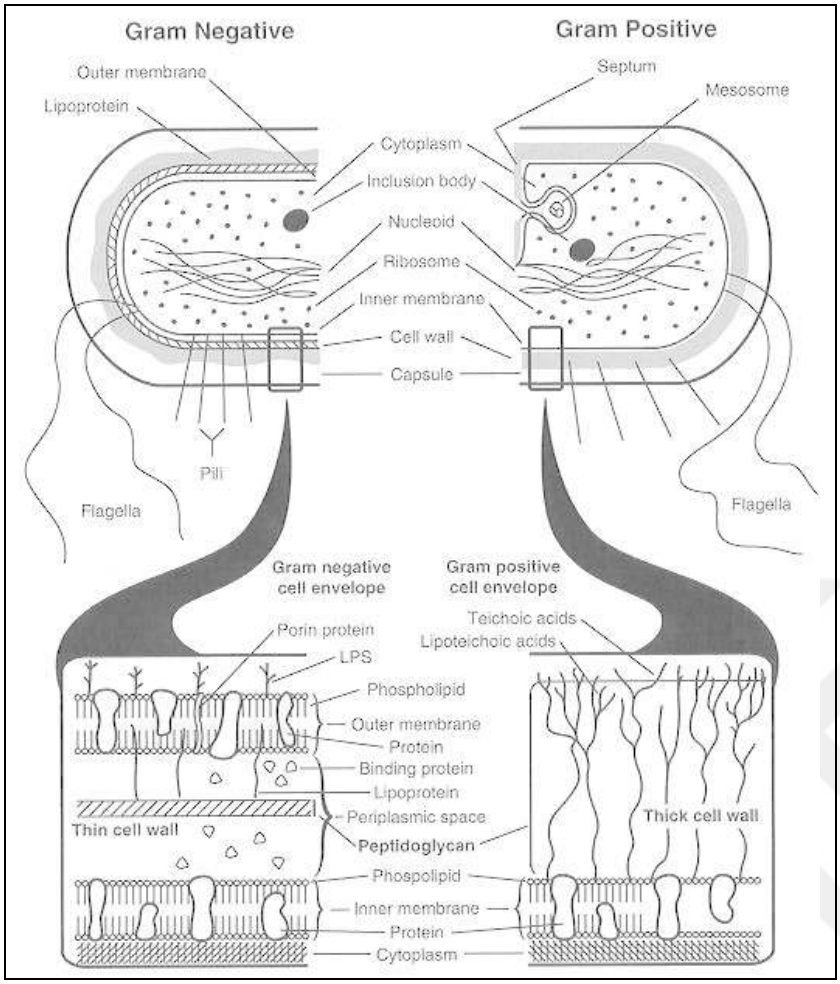


Figure 2.6. Comparison of Gram-positive and Gram-negative bacteria's cell walls. Take note of the intricate structure of the periplasmic space, the outer membrane, and the hydrophobic lipoprotein anchor of the Gram-negative cell envelope (Salton and Kim, 1996)

The cell wall of gram-positive bacteria primarily composed of peptidoglycan has a thickness ranging from approximately 20 to 80 nanometers. This outer layer also hosts other polymers, such as teichoic acids, polysaccharides, and peptidoglycolipids, which are covalently bonded to the peptidoglycan matrix. In contrast, gram-negative bacteria possess a thinner peptidoglycan layer, typically measuring around 5 to 10 nanometers, with *E. coli*, for instance, having a peptidoglycan that is only one layer thick. Following the peptidoglycan layer is a membrane structure measuring 7.5 to 10 nanometers in thickness. This membrane layer is non-covalently attached to lipoproteins, which, in turn, are covalently connected to the peptidoglycan. At the outermost periphery of the gram-negative bacterial membrane resides a sheet composed of lipopolysaccharides (Salton and Kim, 1996), completing the composition of the cell envelope in these bacteria.

2.2.2. Yeast

Yeast, part of the fungus kingdom, differs from bacteria as it is eukaryotic, having a defined nucleus. There are over 1500 identified yeast species (Hoffman et al., 2015; Kurtzman and Fell, 2006; Piškur and Compagno, 2014), with a fungal cell wall that separates the environment from the plasma membrane, offering protection and structural support. This cell wall acts as a flexible skeleton, safeguarding against environmental stresses, including osmotic changes. It mainly consists of glucans, chitin, and glycoproteins (Garcia-Rubio et al., 2020). Figure 2.7 illustrates the outer cell wall of *Candida albicans*, a common yeast species.

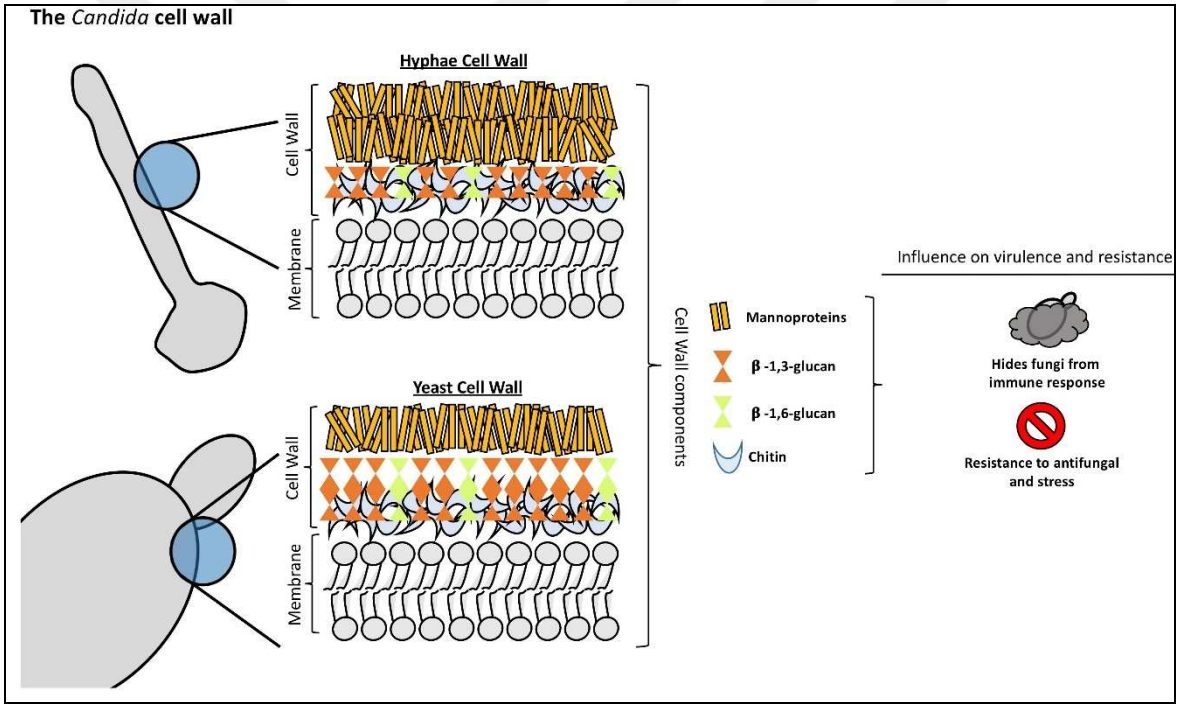


Figure 2.7. Illustration of the cell wall for *Candida albicans* (Garcia-Rubio et al., 2020)

A complex and specialized cellular organelle, the cell wall is made up of glycosylated proteins, chitin, glucans, and chitosan. Glycoproteins are often formed by the association of proteins with polysaccharides. Collectively, these constituents enhance the firmness of the cell wall. Creating and sustaining the cell wall requires the activation of numerous metabolic and signaling pathways (Casadevall and Perfect, 1998; Garcia-Rubio et al., 2020).

In a cell wall, glucan is one of the most important polysaccharides that makes up the structure, constituting 50 to 60 % of the dry weight of the cell wall. Most glucan polymers

consist of glucose units linked by 1,3 bonds, although there are variations with other links like b-1,6 (found in Candida but not in Aspergillus), b-1,4, a-1,3, and a-1,4. The most significant structural component in the wall is b-1,3-D-glucan, to which other components are covalently attached. The b-1,3-D-glucan is produced by glucan synthases, a group of enzymes, which resides in the plasma membrane (Douglas et al., 1994; Garcia-Rubio et al., 2020; Pontón, 2008; Qadota et al., 1996).

What influence the amount of chitin content inside the cell wall of the fungal are the morphological phase. In case where the cell wall is considered, from the total dry weight, only 1 to 2 percent is of chitin. In filamentous fungi, this proportion can increase to 10 to 20 percent. The process in which Chitin is produced is through the enzyme chitin synthase. The process deposits polymers of chitin in the outside of the cell, adjacent to the cytoplasmic membrane (Chattaway et al., 1968; Garcia-Rubio et al., 2020). Proteins comprise approximately 30-50% of the dry weight of yeast cell walls and around 20-30% of the dry weight in filamentous fungi. Many of these proteins are linked to carbohydrates through O or N linkages, resulting in glycoproteins (Bowman and Free, 2006; Garcia-Rubio et al., 2020). Melanin, a high molecular weight pigment, carries a negative charge, is hydrophobic, and remains insoluble in aqueous solutions. It serves as a protective element for fungi, aiding in their survival by shielding them against various stressors, particularly within the host environment (Casadevall et al., 2000; Garcia-Rubio et al., 2020; L. Liu et al., 1999; Nosanchuk et al., 2015; Nosanchuk and Casadevall, 2006).

2.3. Antimicrobial Agents

Antimicrobials are therapeutic substances employed to prevent or treat infections, encompassing categories such as antiseptics, antibiotics, antivirals, antifungals, and antiparasitics. They exert their action by eliminating microorganisms or *Hind*ering their growth, targeting vital aspects of cellular metabolism such as the synthesis of essential biomolecules, the function of cellular enzymes, or the integrity of cellular structures like cell walls and membranes (Di Martino, 2022). Agents designed to kill microorganisms are termed microbicidal, while those that solely impede their growth are referred to as microbiostatic (Ismayil Sumaiya and Nimila, 2019). Antibacterials combat bacterial infections, while antifungals are effective against fungal pathogens (Pandey et al., 2014). The role of antimicrobial agents is pivotal in the control and treatment of infectious diseases.

Nevertheless, these agents face increasing microbial resistance, underscoring the urgency of discovering novel drugs. Numerous studies have indicated the antimicrobial properties of phosphazene derivatives against both bacteria and yeast strains (Mazel and Davies, 1999; Şeker et al., 2018). Given that pathogenic bacteria and yeast strains are central to this thesis research, it is essential to elucidate the characteristics of antibacterial and antifungal drugs.

2.3.1. Antibacterial drugs

Antibacterial drugs, made of chemicals, were developed to fight against infections caused by bacterial pathogens. These drugs are what we know as antibiotics. The development of antibiotics, spanning from 1910 to 2010, has been mainly discussed in (Walesch et al., 2023). The introduction of new antibiotics spanning the century is given in the top blue region in Figure 2.8, including the year they were introduced. The red region below shows some of the bacteria that have been found to be resistant to certain types of antibiotics.

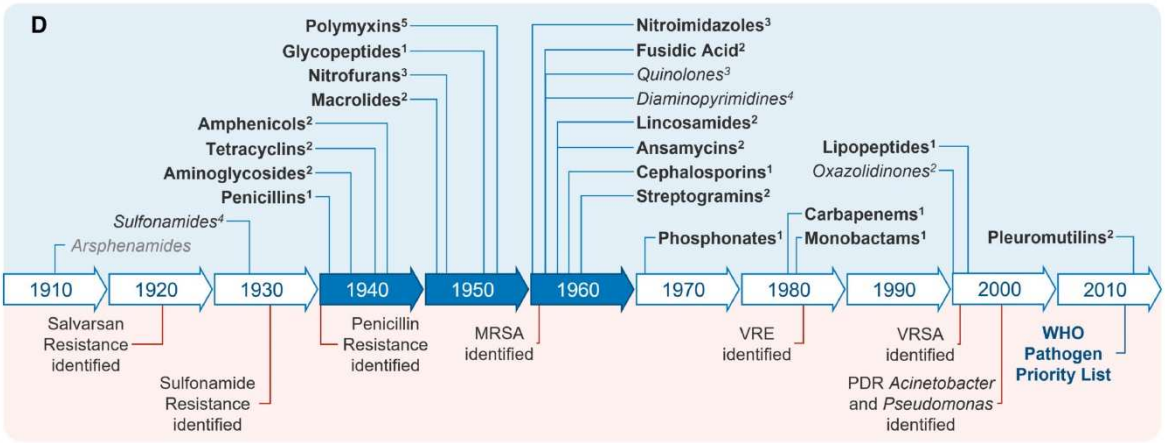


Figure 2.8. Timeline of the introduction of antibiotics (blue) and resistance identification (red) (the image was reprinted by the approval of European Medicines Agency (EMA) (Walesch et al., 2023)

The problem of bacteria becoming antibiotic-resistant is growing with each decade. Now, most antibiotics have been observed to exhibit a certain degree of resistance against certain bacteria. To address this issue, pharmaceutical companies have invested in developing new types of antibiotics to introduce to the market. However, eventually become this development may become saturated as it becomes increasingly difficult to discover new types of antibiotics. With this precedent, the threat of bacterial infections is again becoming relevant, similar to when antibiotics were first introduced (Ventola, 2015).

Antibiotics have different ways in which they work. These mechanisms can be considered methods of classifying antibiotics and are briefly illustrated in Figure 2.9 (Kapoor et al., 2017).

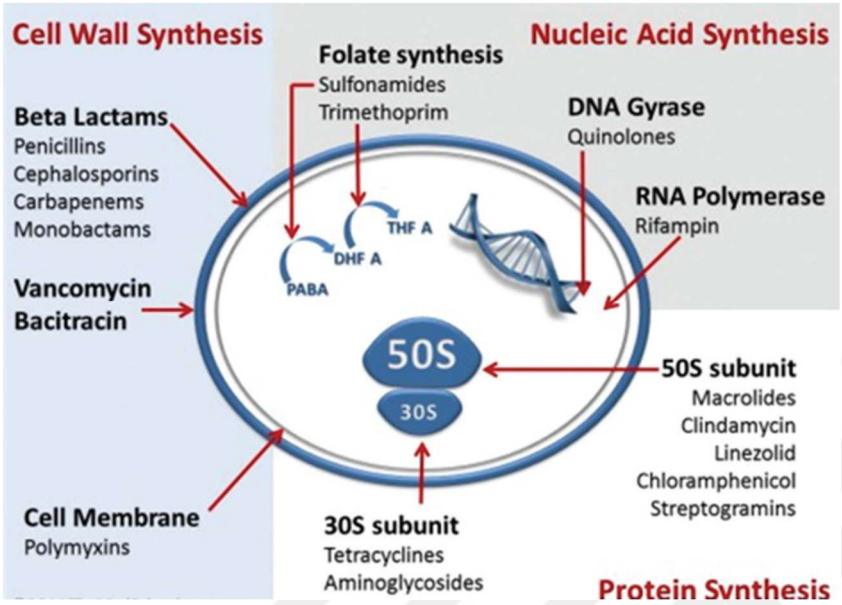


Figure 2.9. Mechanism of action of antibiotics, reprinted from (Kapoor et al., 2017)

Antibiotics that target the cell wall

Examples of these antibiotics include Beta-lactams and Glycopeptides. In the case of Beta-lactams, it is theorized that they prevent the synthesis of new peptidoglycan, a key component of the cell wall. Without the production of the peptidoglycan layer, the bacteria undergo lysis. Glycopeptides, on the other hand, work differently but also result in the inhibition of cell wall synthesis (Kapoor et al., 2017).

Antibiotics that inhibit protein biosynthesis

Antibiotics can disrupt protein biosynthesis occurring in the ribosome. The general mechanism involves inhibiting protein biosynthesis by targeting the bacterial ribosome 70s, consisting of subunits 30s and 50s ribonucleoproteins. Examples of antibiotics that target 30s subunits are Aminoglycosides and Tetracyclines, while examples that target 50s subunits include Macrolides and Oxazolidinones (Kapoor et al., 2017).

Antibiotics that inhibit DNA replication

An example of such antibiotics is Quinolones, which include Fluoroquinolones (FQ). These antibiotics inhibit DNA gyrase activity, subsequently *Hind*ering DNA replication. DNA gyrase comprises subunits A and B, with FQ binding to subunit A, which is responsible for DNA strand cutting and resealing functions (Kapoor et al., 2017).

Antibiotics that inhibit folic acid metabolism

Examples of these antibiotics are Sulfonamides and Trimethoprim. Sulfonamides inhibit folic acid metabolism by targeting dihydropteroate synthase, while Trimethoprim inhibits the dihydrofolate reductase enzyme. Both drugs work in synergy to prevent the production of folic acid at different stages of the process (Yoneyama and Katsumata, 2006).

2.3.2. Antifungal drugs

In the case of fungal infections, several factors must be considered account when choosing treatment. These factors include the host's condition, the location of the infection, and the specific fungi responsible for it. This consideration is essential because different fungi exhibit varying susceptibilities to different types of drugs. Additionally, understanding the pharmacokinetic characteristics of the drug, i.e., how the drug behaves after administration, is crucial.

Antifungal drug research has attempted to classify these drugs into four different classes based on their mechanisms of action: (i) Polyenes, which target ergosterol in the fungal cell membrane, creating channels in the membrane, increasing its permeability, and disrupting the proton gradient (Vanden Bossche et al., 1994). (ii) Azoles, work by inhibiting the production of ergosterol and can also target plasma membrane lipids (Joseph-Horne and Hollomon, 2006). (iii) Echinocandins, which specifically disrupt the biosynthesis of 1,3 β-D-glucan (Denning, 1997). (iv) Flucytosine, which disrupts protein (Polak and Scholer, 1975) and DNA synthesis (Diasio et al., 1978). A brief overview of this classification, pharmacology, the antifungal targets, and the mechanisms of action is provided in Table 2.2.

Table 2.2. Some antifungal agents, their targets, and mechanisms of action (Akkuş and Kaçmaz, 2023)

Antifungal Agent	Pharmacology	Target	Mechanisms of Action
Polyenes	Amphotericin B deoxycholate (ABD)	Synthesis of ergosterol	It binds to the fungal cell wall ergosterol, increasing cell wall permeability
	Amphotericin B lipid complex (ABLC)		
	Liposomal amphotericin B (LAMB)		
Azoles	Fluconazole	Synthesis of ergosterol	Inhibit the enzyme lanosterol-14α-demethylase
	Voriconazole		
	Itraconazole		
	Posaconazole		
	Isavuconazole		
Echinocandins	Caspofungin	Cell wall synthesis	(1→3)-β-d glucan synthase inhibitor
Flucytosine	5-fluorocytosine	Nucleic acid synthesis and hence protein synthesis	Pyrimidine analog that is transported into fungal cells via the enzyme cytosine permease and converted into fluorouracil by cytosine deaminase

2.3.3. Antimicrobial resistance

Bacteria, fungi, viruses, and parasites, may evolve to become resistant to medication. This phenomenon is called antimicrobial resistance (AMR). It has become a significant concern, with many seminal papers and related organizations referring to it as the 'silent pandemic' (Tang et al., 2023; WHO, 2023). This is because its global impact may not be readily apparent, but the potential devastation it could cause is severe. The problem has become one of the most concerning issues of this century, as the number of AMR cases continues to mount, while the development of new medications is not progressing rapidly (Prestinaci et al., 2015).

The rise of deaths related to AMR in 2019 was predicted to go up 1.2 million or more, and, if no considerable measure is taken until 2050, this number may rise to 10 million per year (O'Neill, 2016). This alone would attribute AMR as the worlds's primary cause of death

(O'Neill, 2016). Given this situation, the goal of current work is part of an endeavor to find a new drug that can mitigate the problem within the scope of bacteria and yeast.

Antibacterial resistance

The drugs used to combat bacterial infections are called antibiotics. The susceptibility or resistance of bacteria to a specific type of drug can be expressed in terms of its Minimum Inhibitory Concentration (MIC). MIC is defined as the minimum concentration at which a drug will stop the growth of a particular bacterium (Reygaert, 2018). Whether bacteria are susceptible or resistant to a drug depends on the range within which the average MIC falls. Discovering the average MIC will lead us to the question of how certain bacteria are resistant to particular antibiotics in the first place.

Resistance of bacteria to drugs can occur naturally. This type of resistance is classified as either intrinsic or induced. Intrinsic resistance means that the resistance is always present within the species itself. Intrinsic resistance would mean that the resistance mechanism is current for the genus or species of the bacteria, not preventing the specific antibiotic in general (Courvalin, 2008). In the case of induced resistance, the resistance mechanism is always there but is only expressed after exposure to the antibiotic. In contrast to natural resistance, acquired resistance is obtained when genetic material from related organisms is acquired through any type considered as horizontal gene transfer (HGT), which includes transformation, transposition, and conjugation. In addition, resistance may occur due to mutations in its chromosomal DNA (Reygaert, 2018).

Natural resistance (intrinsic and induced) and acquired resistance are two different ways bacteria develop resistance mechanisms. However, the mechanisms by which the bacteria deter antibiotics do not differ between the two. These mechanisms, as described by Courvalin (Courvalin, 2008), include: (i) Reducing (or eliminating) the drug's affinity to reach the target within bacteria; (ii) Producing enzymes that detoxify the drug; (iii) Increasing impermeability; (iv) preventing the drug from passing through the cell membrane and expelling the drug through energy-dependent pumps. These four major pathways of the resistance mechanism can occur due to either natural resistance or acquired resistance, or a combination of both (Courvalin, 2008).

Another terminology relevant to describing resistance based on the genetics of the bacteria is endogenous and exogenous resistance. Endogenous resistance arises due to mutations and the selection of chromosomal genes within the pathogen itself (Silver, 2011). It is generally not infectious between bacteria (Courvalin, 2008). Exogenous resistance, on the other hand, arises from horizontal gene transfer (HGT) among bacteria, allowing them to inherit genetic elements from their environment. Table 2.3 briefly summarizes various mechanisms of endogenous and exogenous resistances with examples of multiple drugs affected by the particular mechanism.

Table 2.3. Antibacterial resistances, their origin, mechanisms and examples (Silver, 2011)

Origin	Mechanism	Examples of affected drug classes
Endogenous	Single mutations reducing target affinity	Rifamycin, streptomycin, trimethoprim (for Gram-positive organisms), fusidic acid
	Multistep mutations reducing affinity or remodeling of target	Fluoroquinolones, oxazolidinones, daptomycin, vancomycin, polymyxin, β-lactams (for transformable species)
	General efflux mechanism	Most classes for Pseudomonas; many classes for other species
	Reduced uptake (porin or permease loss)	Carbapenems, fosfomycin
	Loss of activation	Metronidazole (for *H. pylori*)
	Upregulation of target	Fosfomycin
Exogenous	Class-specific efflux	Tetracycline, macrolides
	Class-specific degradation/modification	β-Lactams, aminoglycosides, chloramphenicol, streptogramin A, metronidazole (for anaerobes), fosfomycin
	Target protection/modification	Tetracycline, macrolides, lincosamides, oxazolidinones, streptogramin B
	Replacement with reduced-affinity target	β-Lactams, vancomycin, trimethoprim, mupirocin, sulfonamides
	Sequestration of target	Fluoroquinolones, fusidic acid

Antifungal resistance

The threat caused by fungi ranges from superficial to dangerous, such as that caused by invasive fungal disease (IFD). It is predicted that one billion people are affected worldwide by fungi-related causes. As mentioned in Section 2.3.2, four main classes of drugs are used

to treat fungal-related diseases: polyenes, azoles, echinocandins, and flucytosine. Treatment with these chemical drugs has seen cases of inefficacy in response to the disease. It may be that the failure because the fungi have built antifungal resistance by adapting to the drug as they are exposed to it. Antifungal resistance means that the fungi are not susceptible to the drug anymore and can still grow even in the presence of a high drug concentration (Fisher et al., 2022).

Ferrari and Sanglard (Ferrari and Sanglard, 2010) compiled studies on antifungal resistance and their mechanisms. Their review was summarized in a table, similar to what is shown in Table 2.4. The main mechanisms that were reported consist of: (i) impaired transport, related to the efficacy of transport mechanisms within the cell; (ii) target alteration, related to the reduced affinity of the drug for the compatible target; (iii) absence of target, related to the lack of the compatible target designed for the drug to work; (iv) decreased toxic metabolite, related to the reduced toxicity of the drug regarding the fungi; (v) formation of multicellular complexes, related to the formation of larger structured communities of the fungi, e.g., biofilms.

Table 2.4. Reported resistance and the mechanisms of the resistance of major systemic antifungal agents (Ferrari and Sanglard, 2010)

Antifungal agent	Reported resistance mechanism				
	Impaired transport	Target alteration	Absence of Target	Decrease toxic metabolite formation	Formation of multicellular complexes
Polyenes (Amphotericin B)			ERG6 and ERG11 inactivation		Formation Biofilms
Azoles (Itraconazole, Fluconazole, Voriconazole, Posaconazole)	ABC-transporter and Major Facilitator upregulation	ERG11 mutation and upregulation	ERG11 inactivation	ERG3 nonsense mutation	Formation Biofilms

Table 2.4. Reported resistance and the mechanisms of the resistance of major systemic antifungal agents (Ferrari and Sanglard, 2010) (continued)

Antifungal agent	Reported resistance mechanism				
	Impaired transport	Target alteration	Absence of Target	Decrease toxic metabolite formation	Formation of multicellular complexes
Flocytocine (5-FC)	Permease (FCY2) mutation			FUR1 mutation	Formation Biofilms
Echinocandins (Caspofungin, Micafungin, Anidalafungin)		FKS1 and FKS2 mutation			

2.4. Cancer

A class of disorders known as cancer is defined by the unchecked division and dissemination of aberrant cells to other body areas. Numerous factors, including genetic mutations, environmental influences, and lifestyle decisions, have been linked to the causes of cancer (National Cancer Institute, 2021). The cancer disease has been attributed as the second in terms of cause of death. This amounts to as much as 10 million deaths in in 2020 based on WHO report. The leading cancer types contracted by males are stomach and liver cancers, and for females, they are breast, colorectal, lung, cervical, and thyroid cancers (Siegel et al., 2023). Cancer patients may receive chemotherapy, radiation therapy, and cancer medications, singly or combined.

2.4.1. Cancer cell

Some key characteristics of cancer cells include (Ruddon, 2003): (i) Uncontrolled growth at an abnormal rate; (ii) Evasion of the immune system allowing it to spread without detection within the host; (iii) Metastasis, which allow cancer to spread to a different part of the body, forming secondary tumor; (iv) Genetic mutations contributing to the abnormal growth; (v) The inability to differentiate cell types, leading to tumors of mix cell types; (vi) Different method of energy production, promoting ways to the uncontrolled growth. Understanding the characteristics of cancer cells is crucial for developing effective treatments and

prevention strategies. Researchers study cancer cells to identify the specific genetic and molecular changes that contribute to their uncontrolled growth and division, and their ability to evade the immune system and spread to other parts of the body (Ruddon, 2003).

2.4.2. Formation of cancer cells

Cancer cell formation begins when normal cells undergo genetic mutations that disrupt their regulatory mechanisms. Some of these genetic alterations can transform the healthy genes that regulate cell growth, division, and differentiation called proto-oncogenes, into oncogenes. Oncogenes are genes that promote uncontrolled cell division and growth (Chial, 2008). Explaining these two genes will provide insight into cancer cell formation.

Many oncogenes start as proto-oncogenes, typical genes responsible for regulating cell growth, proliferation, and preventing apoptosis (Yokota, 2000). These genes are involved in producing essential proteins for various bodily functions and are instrumental in processes like cell division. Typically, proto-oncogenes carry out their functions smoothly. Nevertheless, when a mutation arises within a proto-oncogene, it becomes persistently active, resulting in an overproduction of proteins that promote cell growth (Cafasso and Collins, 2022). Two examples of proto-oncogenes that can develop into oncogenes are Ras and RET. Ras encodes an intracellular signal-transduction protein and is involved in cell growth. When it mutates, it encodes for a protein that causes an uncontrolled growth of cells (Cafasso and Collins, 2022). RET encodes a receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor (GDNF) family of extracellular proteins (Knowles et al., 2006).

2.4.3. Cancer types

There are many types of cancer, can be classified based on various criteria, such as the type of cell they start in or the location in the body where they develop. Some common types of cancer include (Cancer Research UK, n.d.; Centers Prevention of Disease Control, n.d.; National Cancer Institute, n.d.): (i) Carcinomas, cancer that affect the skin or tissues that cover internal organs such as breast, lung, bowel, and prostate; (ii) Sarcomas, cancer that develop in connective tissue such as bone, cartilage, and muscle; (iii) Leukemias, cancer of the blood or bone marrow which can be seen by the abnormal increase of white blood cell;

(iv) Lymphomas, cancer in lymphatic system; (v) Gynecologic cancer, cancer that affect woman's reproductive organs (cervix, ovaries, uterus, vagina, vulva); (vi) Melanoma, cancer of the skin pigment; (vii) Pancreatic cancer, cancer that affect the pancreas; (viii) Prostate cancer, cancer in men's prostate. Many types of cancer that can affect the human body other than what has been mentioned here. Each type of cancer has its unique characteristics, risk factors, and treatment approaches.

2.4.4. Medications used in cancer treatment

There are various medications used in cancer treatment, and the choice of drugs depends on the type of cancer, its stage, and the individual patient's health. Some common types of medications used in cancer treatment include (Ames, 2021): (i) Chemotherapy drugs that is design to kill cancer cells or stop them from multiplying (examples: paclitaxel, carboplatin, and fluorouracil); (ii) Target therapy drugs that target abnormalities within cancer cells (examples: trastuzumab, imatinib, and vemurafenib); (iii) Hormone therapy drugs that change the way hormones work or interfere with the body's ability to produce them, to help slow or stop the growth of hormone-sensitive tumors (examples: tamoxifen, letrozole, and leuprolide); (iv) Immunotherapy drugs that help the immune system to attack cancer cells (examples: pembrolizumab, nivolumab, and ipilimumab); (v) Bone modifying drugs that prevent or slow down the weakening of the bones, used to when cancer spreads to the bone (examples: zoledronic acid and denosumab); (vi) Radiopharmaceuticals which contain small amount of radiation used to treat thyroid cancer and some types of bone cancer, to name a few.

The mechanisms of action of clinically used chemotherapy involve various processes, including the induction of DNA damage, inhibition of macromolecular synthesis, and activation of cell death pathways. Chemotherapy drugs can affect normal cells, leading to toxic effects, and are commonly associated with side effects reflecting their mechanism of action. Patients may experience chemotherapy differently, mainly because of how the host's cells have their own sensitivity and resistance towards the cytotoxic effects of the drug. The clinically established types of chemotherapy are classified into alkylating agents, antimetabolites, topoisomerase inhibitors, antibiotics, mitotic inhibitors, and protein kinase inhibitors, all of which have different mechanisms to work against cancerous cells. The therapeutic response to chemotherapy remains limited. What has gained popularity in this

topic is the use of a variety of chemotherapy, in combination. The goal is that the different mechanisms of the drugs may work in conjunction with each other to improve drug uptake and efflux. Additionally, chemotherapy agents are commonly associated with side effects, and their efficacy is influenced by various individual and cancer-related factors (Amjad et al., 2023).

2.5. DNA Molecule and Structure

The purpose of DNA is to carry genetic information by being involved in the synthesis of proteins that would then allow for a cell to perform metabolic activities and maintain life (Klug et al., 2006). The well-known double-stranded and superhelical model of DNA was initially disseminated by the pair of author, James Watson and Francis Crick, in 1953 (Watson and Crick, 1953). What they proposed is a model of DNA that forms a linear polymer consisting of nucleotides that are sequenced in a specific manner. These nucleotides are composed of three distinct components: (i) a 5-carbon sugar called deoxyribose; (ii) a phosphate group; (iii) an organic nitrogenous base. The latter organic base can be further categorized into Purines (double-ring structure of adenine, A, and guanine, G) and Pyrimidines (single-ring structure of cytosine, C, and thymine, T) (Klug et al., 2006; Watson and Crick, 1953).

Nucleotides are the building blocks of a longer nucleic acid chain linked by a phosphodiester bond. The way this bond is arranged is based on a specific orientation: the 3’ hydroxyl group (-OH attached to the 3’ carbon) of one sugar to the 5’ phosphate group (attached to the 5’ carbon) of another (Watson and Crick, 1953; Wing et al., 1980). The illustration for these bonds is given in Figure 2.9. Additionally, one can see the difference in the number of bonds that links: (i) cytosine (symbolized as C in Figure 2.9) and guanine (G); (ii) adenine (A) and thymine (T). The former are connected together by three hydrogen bonds, whereas the latter are connected by two hydrogen bonds.

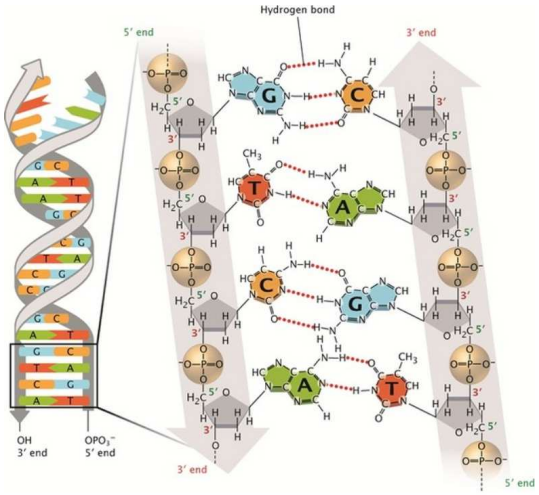


Figure 2.10. DNA structure the 5' and 3' end with A, G, C and T refers to Adenine, Guanine, Cytosine, and Thymine respectively (Pray, 2008)

2.5.1. The mechanism of DNA targeting drug

A drug targeting DNA may provide an effective treatment method considering that DNA transcription and replication are vital processes within a cell. The potential for the cancer treatment by DNA-targeting drugs arises from the reasoning that many of the molecules within the cell itself exhibit anticancer properties when interacting with DNA, which could potentially prevent the development of tumors. Thus, it is imperative to understand, when designing this type of drug, the intricacy of the interaction between DNA and molecules within the cell and how it affects tumor and cancer development to produce an effective anticancer drug (Reinhold et al., 2017). An example of two different classes of drugs that target DNA is given below:

Alkylating agents

Alkylating agents are substances that form covalent bonds by reacting with atoms in biological molecules with abundant electrons (Colvin, 2003). This covalent binding inhibits DNA processes, such as replication, transcription, RNA, and protein synthesis (Yuhas, Majumdar, and Greenberg, 2021). Alkylating agents may be produced by the host internally (in case of oxidative stress due to disease) or through external means (water, fuel, air, tobacco products, etc.). When exposed to the host, these alkylating agents may damage healthy cells due to cytotoxic, carcinogenic, and teratogenic effects. However, its property to damage cell can be used as chemotherapeutics when dealing with cancer cells. Some

examples of alkylating agents that have been used clinically are ethylenamine, busulfan, and cisplatin (National Institute of Diabetes and Digestive and Kidney Diseases, 2015). Cell death caused by alkylating agents works through covalent binding with DNA.

Intercalating agents

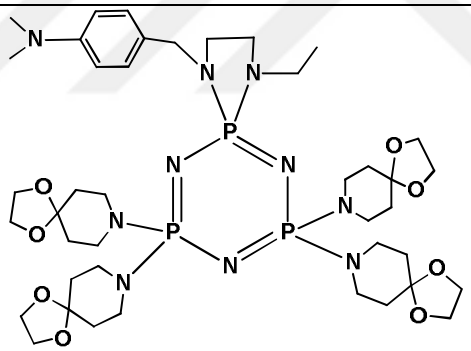
DNA molecules exhibit reversibility, allowing them to join and separate as needed. The connection between these molecules relies on non-covalent interactions, which differ from covalent bonds in that they don't involve sharing electrons. Intercalating agents use this type of connection to insert themselves between adjacent bases in a DNA strand, resulting in various deformations, such as kinking or uncoiling of the DNA. This can give rise to frameshift mutations during the replication process, leading to either deletions or insertions in the DNA sequence. Intercalating agents are commonly used as fluorescent markers for visualizing DNA. Examples of these agents include ethidium bromide, acridine orange, and proflavine.

3. MATERIALS AND METHODS

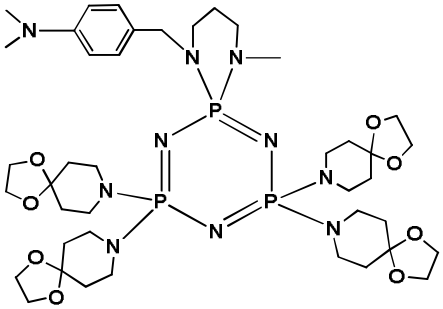
3.1. Materials

3.1.1. Phosphazenes

The phosphazene derivatives used in the research were synthesized by the group at Ankara University, Faculty of Science, in their Chemistry Laboratories, by Prof. Dr. Zeynel KILIÇ and Prof. Dr. Aytuğ OKUMUŞ, as well as by Dr. Güler İnci Tanrıkulu in the Chemistry Laboratories of the Faculty of Science at Amasya University. The molecular-structure of the five different phosphazene compounds, referred to as 26, 27, 28, 29, and 30 in the study, is provided in Figure 3.1. Dimethyl sulfoxide (DMSO) was used to dissolved the compounds. The mixtures were then sterilized by running them through a 0.45 μm pore size injector tip filter.

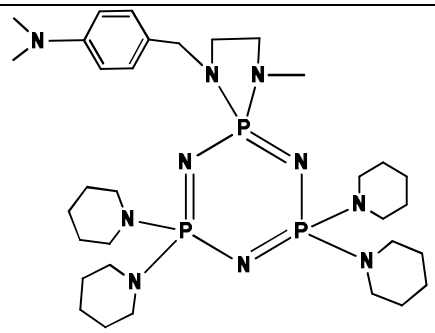


(Compound 26)
1-[(4-dimetilaminobenzil)metil]-4-etil-7,7,9,9-tetra-(1,4-dioksa-8-azaspiro[4,5]dekano)-1,4,6,8,10- pentaaza-5λ^5, 7λ^5, 9λ^5 -trifosfaspiro[4.5]deka-5,7,9-trien

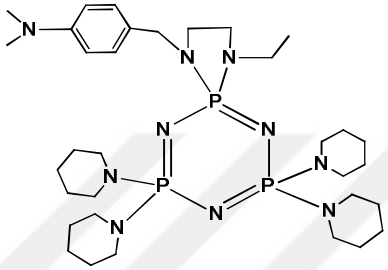


(Compound 27)
7-[(4-dimetilaminobenzil)metil]-11-metil-2,2,4,4-tetra-(1,4-dioksa-8-azaspiro[4,5]dekano)-1,3,5,7,11- pentaaza-2λ^5, 4λ^5, 6λ^5 -trifosfaspiro[5.5]undeka-1,3,5-trien

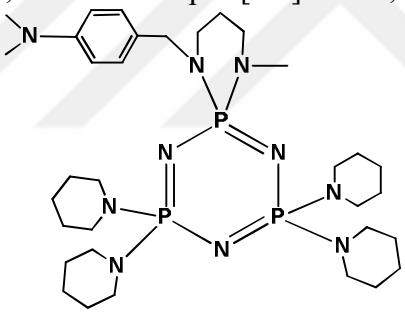
Figure 3.1. Compounds used in the study



(Compound 28)
1-[(4-dimetilaminobenzil)metil]-4-metil-7,7,9,9-tetra-1-piperidinil-1,4,6,8,10- pentaaza-5λ^5, 7λ^5, 9λ^5 -trifosfaspiro[4.5]deka-5,7,9-trien



(Compound 29)
1-[(4-dimetilaminobenzil)metil]-4-etil-7,7,9,9-tetra-1-piperidinil-1,4,6,8,10- pentaaza-5λ^5, 7λ^5, 9λ^5 -trifosfaspiro[4.5]deka-5,7,9-trien



(Compound 30)
7-[(4-dimetilaminobenzil)metil]-11-metil-2,2,4,4-tetra-1-piperidinil-1,3,5,7,11-pentaaza-2λ^5, 4λ^5, 6λ^5 -trifosfaspiro[5.5]undeka-1,3,5-trien

Figure 3.1. Compounds used in the study (continued)

3.1.2. Microorganisms and growth media used

This research used 11 bacterial strains and 3 yeast strains to detect the antimicrobial activity of the compound. These microbes were retrieved from the collection of cultures in the Molecular Biology Laboratory, Faculty of Science, Gazi University (See Table 3.1). These microbes consist of a variety of gram-positive and gram-negative bacteria alongside yeast, all of which have been presented as well in Table 1.1. All microorganisms included in the study were first transferred from stock and activated under appropriate incubation conditions. Bacteria were activated in Nutrient Agar (NA) medium, and yeast in Sabouraud

Dextrose Agar (SDA) medium under suitable incubation conditions.

Table 3.1. Microorganisms used in the study and incubation conditions

Microorganisms	Incubation Temperature	Incubation Time	Growth Medium
Escherichia coli ATCC 35218	37 °C	24 hours	NA
Escherichia coli ATCC 25922	37 °C	24 hours	NA
Bacillus cereus NRRL B-3711	37 °C	24 hours	NA
Bacillus subtilis ATCC 6633	37 °C	24 hours	NA
Staphylococcus aureus ATCC 25923	37 °C	24 hours	NA
Enterococcus faecalis ATCC 29212	37 °C	24 hours	NA
Pseudomonas aeruginosa ATCC 27853	37 °C	24 hours	NA
Klebsiella pneumoniae ATCC 13883	37 °C	24 hours	NA
Salmonella typhimurium ATCC 14028	37 °C	24 hours	NA
Enterococcus hirae ATCC 9790	37 °C	24 hours	NA
Proteus vulgaris RSKK 96029	37 °C	24 hours	NA
Candida albicans ATCC 10231	30 °C	48 hours	SDA
Candida krusei ATCC 6258	30 °C	48 hours	SDA
Candida tropicalis NRRL Y-12968	30 °C	48 hours	SDA

3.1.3. Chemicals used

Nutrient Agar (Merck), Sabouraud Dextrose Agar (Merck), Muller Hinton Agar (Merck), Mueller Hinton Broth (Merck), Sabouraud Dextrose Broth (Merck), NaCl (Merck), Dimethyl Sulfoxide (DMSO) (Sigma), Tris (Merck), acetic acid (Merck), ethylenediaminetetraacetic acid (EDTA) (Merck), agarose (Applichem), ethidium bromide (Sigma), ethanol (Merck), sucrose (Sigma), bromophenol blue (Merck), Tris-EDTA (TE).

3.1.4. Consumables, tools, and equipment used

Petri dishes (90x15 mm) (LP Italiana), micropipette tips (LP Italiana), syringe filter tip (0.45 µm) (Sartoirus), various glass materials such as Erlenmeyer flasks, beakers, measuring cups, latex and nitrile gloves, inoculation loop (ose), cotton, 96-well microplate, various volumes

of micropipettes, autoclave (Tomy SX-500), incubator (37°C) and incubator (40°C), kit storage cabinet (+4°C) (Sanyo), deep freezer (-30 and -80°C) (Sanyo), gel imaging device (Biometra), power supply, pH meter (Mettler Toledo), precision balance, sterile hood, refrigerated centrifuge (Sigma), horizontal electrophoresis system, vortex, microwave oven (Samsung).

3.1.5. Solutions preparation

Physiological saline:
Physiological saline with 0.9% NaCl was prepared by dissolving NaCl in distilled water. The solution was then sterilized heating it inside an autoclave with temperature of 121°C under pressure of 1.5 atm for 15 minutes.

Culture Media for Bacteria and Yeast:
The sterilized medium was dissolved in distilled water according to the recommended amount by the manufacturer and cooled to a temperature of 60°C. Then, the solid medium was poured into sterile petri dishes in aseptic conditions, about 18-22 ml each, and the liquid media was transferred into 10 ml glass tubes. After the solid medium solidifies and the liquid media cools down, it was then incubated overnight in an incubator for sterility control. Afterward, it was stored in a +4°C refrigerator until used in the research.

Sterilization Process:
Sterilization was performed using an autoclave at 121°C under 1.5 atm pressure for 15 minutes.

Tris-acetate EDTA (TAE) Buffer (50X):
Three different components, namely, 242 g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5M EDTA (with a pH of 8) were dissolved together with distilled water at a small amount. The final product had a volume of 1 liter after adjustment.

0.5M EDTA (pH 8.0):
Starting from 186.12 g of EDTA, it then dissolved into a small amount of distilled water. The addition of NaOH tablets adjusted the pH to 8, and the volume was adjusted to 800 ml.

Then, it was mixed until the pH reached 8. Distilled water was then added to get 1 of the final product.

1 M Tris-HCl:
An amount of 12.11 g of Tris was weighed, dissolved in a small amount of distilled water. Adjustment of the pH was done by adding concentrated HCl until it reached 8. Then, distillied water was added to adjust the final volume to 100 ml.

TE (Tris-EDTA) Buffer:
The combination of 1 ml of 1 M Tris-HCl and 0.2 ml of 0.5 M EDTA at the same pH of 8 was mixed, and distilled water was added to adjust the volume to 100 ml of the solution.

TAE Buffer:
The buffer was made by taking 20 ml of 50X TAE. Adjustment was made until the volume was 1 liter with distilled water.

Agarose Gel:
1% (w/v) agarose was dissolved in 1X TAE buffer, boiled in a microwave, then cooled for some time before being poured into a gel plate. The gel was left to solidify.

Gel Loading Buffer:
It was prepared with 40% sucrose, 0.025% bromophenol blue, and 0.25% xylene cyanol.

Ethidium Bromide:
The solution was prepared for a concentration of 10 mg/ml and stored in dark-colored bottles.

3.2. Methods

3.2.1. Determination of the antimicrobial activity of compounds

Adjustment of microorganism concentration according to McFarland standard

The inoculation of the bacteria was done on petri dishes which contain Muller Hinton Agar, taken from stock cultures through a single colony inoculation. This was then followed by an incubation for 24 hours at 37°C. After incubation, the actively growing bacterial cultures were transferred to sterile tubes containing physiological saline, adjusted to a density of McFarland No: 0.5 (measured by colony formuing unit, $1x10^8$ cfu/ml).

Similarly, inoculation of yeast cells was done on petri dishes containing SDA. The incubation of the yeast cells were done for 48 hours at 30°C. The actively growing yeast cultures were then transferred to sterile tubes containing physiological saline, adjusted to a density of McFarland No: 0.5 ($1x10^7$ cfu/ml).

Microdilution assay

The activity of the phosphazene compounds, measured in MIC and MBC/MFC, was obtained from the method of microdilution. Liquid culture media either Sabouraud Dextrose Broth or Mueller Hinton Broth were distributed to a 96-well microplate, each receiving 100 µl. A compound with a concentration of 5000 µM was taken, and 100 µl was added to the first well. From the first well, the same amount was transferred to the next well, and this process was repeated, achieving varying concentrations of the compounds, which ranged from 2500 to 19.54 µM at a 1/2 dilution ratio. Based on reference, the antibacterial agents ampicillin (10 mg/mL) and chloramphenicol (30 mg/mL) were chosen for the positive control, whereas the antifungal agent ketoconazole (50 mg/mL) was chosen. Microorganisms, adjusted to McFarland No: 0.5 ($1x10^8$ colony-forming units (cfu)/ml), were diluted 1:10 in physiological saline, and 5 µl were added to each well. Only the culture medium was used as negative control, and as positive control, the culture medium with microorganisms was used. Microplates with bacteria were incubated for 24 hours at 37°C, and plates with yeast were incubated for 48 hours at 30°C. After incubation, 10 µl samples were taken from each well in the microplates. For bacteria, the samples were streaked onto

Mueller Hinton agar, and for yeast, they were streaked onto Sabouraud Dextrose Agar. After appropriate incubation periods, the growth of microorganisms at different concentrations was checked. The lowest concentration where there was no microbial growth was considered the minimum bactericidal concentration (MBC) for bacteria and the minimum fungicidal concentration (MFC) for yeast. After incubation, the first concentration at which microbial growth slowed down was considered the minimum inhibitory concentration (MIC).

3.2.2. The interaction of DNA and compounds

Using agarose gel electrophoresis, the effects of phosphazene compounds 26, 27, 28, 29, and 30 on plasmid DNA were examined. Dimethyl sulfoxide (DMSO) stock solutions of the compounds were made and used within an hour. Following a 24-hour incubation period at 37°C, compounds containing plasmid DNA at concentrations of 5000, 2500, 1250, 625, and 312.5 μM were loaded onto an agarose gel and the formed bands were analyzed. Plasmid DNA that was unaffected by the chemical was inserted into the gel's first well as a control.

Agarose gel electrophoresis

In a gel tray, 1% (w/v) agarose solution in TAE buffer was added, and the mixture was left to polymerize. Following polymerization, the gel was divided into wells, and loading buffer and drug-DNA were added to these wells. For three hours, electrophoresis was performed in TAE buffer at 70 V. The gel was stained with ethidium bromide after electrophoresis. The gels were then seen under ultraviolet light using the BioDoc Analyze (Biometra) gel imaging apparatus, and the pictures were stored on a computer in JPEG format.

*Bam*HI and *Hind*III restriction enzyme reaction

*Bam*HI enzyme recognizes the 5'-G/GATCC-3' region on DNA, while *Hind*III enzyme recognizes the 5'-A/AGCTT-3' region. To understand the binding of compounds to these regions on DNA, the compounds and DNA were incubated for 24 hours. Subsequently, they were treated with *Bam*HI and *Hind*III enzymes and further incubated for 1 hour at 37°C. The drug-DNA mixture that had been treated with enzymes was run for one hour at 70 V in TAE buffer on a 1% agarose gel. Following electrophoresis, the gel was stained with ethidium

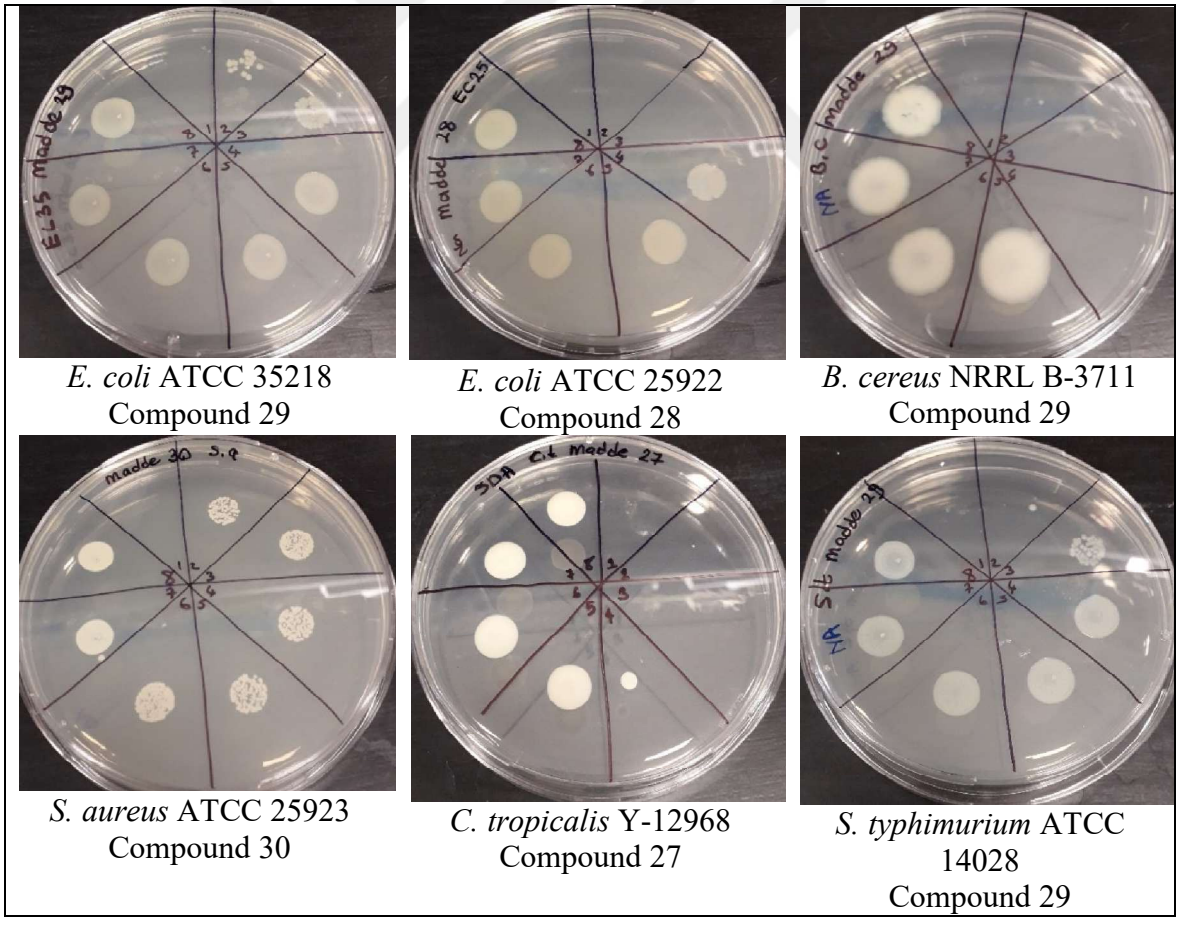
bromide and the BioDoc Analyze (Biometra) gel imaging instrument was used to view the gels under ultraviolet light. Images were then saved in JPEG format on a computer.

4. RESULTS OF RESEARCH

4.1. In Vitro Antimicrobial Activity

Compounds 26, 27, 28, 29, and 30 were tested for their antimicrobial activity at a concentration of 5000 µM, determined using a ½ dilution ratio, resulting in compound concentrations ranging from 2500, 1250, 625, 312.5, 156.3, 78.1, 39.1, and 19.54 µM. Antibiotics (ampicillin and chloramphenicol) and antifungals (ketoconazole) were used as positive controls based on reference.

After the incubation period ended, the visual growth produced by the compounds against several microorganisms was observed and depicted in Image 4.1.



E. coli ATCC 35218
Compound 29

E. coli ATCC 25922
Compound 28

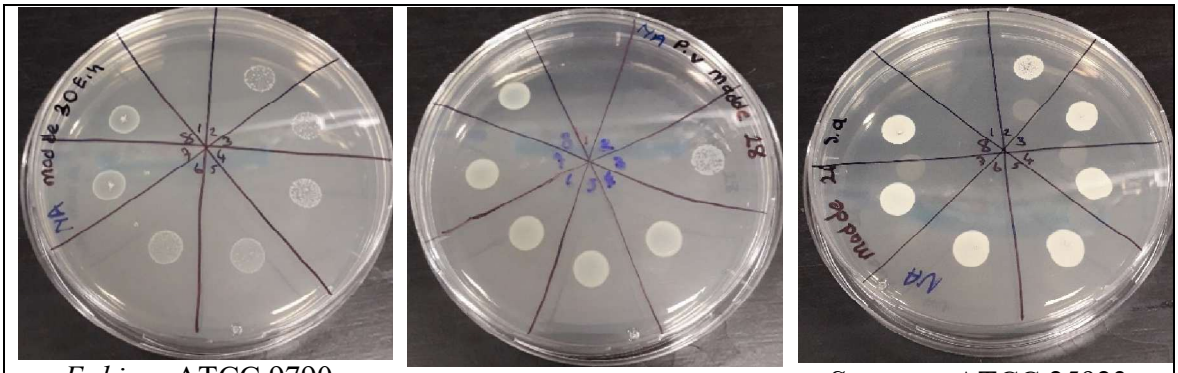
B. cereus NRRL B-3711
Compound 29

S. aureus ATCC 25923
Compound 30

C. tropicalis Y-12968
Compound 27

S. typhimurium ATCC 14028
Compound 29

Image 4.1. Visual growth created by compounds against several microorganisms



E. hirae ATCC 9790
Compound 30

P. vulgaris RSKK 96029
Compound 28

S. aureus ATCC 25923
Compound 26

Image 4.1. Visual growth created by compounds against several microorganisms (continued)

MIC values for compounds 26, 27, 28, 29, and 30 were determined using the microdilution method, while antibacterial drugs (ampicillin, chloramphenicol) and antifungal drugs (ketoconazole) were used as positive controls based on references. In addition, MBC and MFC values were also determined using the microdilution method. The MIC, MBC, and MFC values found varied from (<19.54) to (>2500 µM).

The observed MIC values from the research results are presented in Table 4.1. MIC values of compounds that demonstrate potential as growth inhibitors for the respective microbe are marked in yellow. A compound's potential was evaluated by comparing its MIC value to that of the positive control. If the MIC value of a compound was found to be lower than the MIC value of the positive control, then that compound is considered to have potential as an inhibitor, and its MIC value is marked in yellow. Positive control data was retrieved from (Cemaloğlu et al., 2023).

The MIC value of compound 26 against *E. coli* ATCC 35218 was 1250 µM; *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 13883 were 625 µM. The effect of compound 26 on *P. aeruginosa* ATCC 27853 was higher compared to that on *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 13883.

The MIC value of compound 27 against *P. aeruginosa* ATCC 27853 and *P. vulgaris* RSKK 96029 was 625 µM. The effect of compound 27 on *P. aeruginosa* ATCC 27853 was higher than on *P. vulgaris* RSKK 96029.

Table 4.1. MIC (reported in µM) of the compounds and positive controls against test strains

MIC								
Compounds						Positive Controls		
Test Microorganisms	26	27	28	29	30	AMP	C	K
E. coli ATCC 35218 G(-)	1250	2500	625	625	1250	2500	625	-
E. coli ATCC 25922 G(-)	2500	625	312.5	625	2500	<19.5	78.1	-
B. cereus NRRL B-3711 G(-)	625	>2500	625	312.5	1250	156.3	156.3	-
B. subtilis ATCC 6633 G(+)	>2500	1250	>2500	1250	1250	<19.5	78.1	-
S. aureus ATCC 25923 G(+)	1250	1250	1250	625	78.1	<19.5	156.3	-
E. faecalis ATCC 29212 G(+)	625	625	625	625	625	<19.5	312.5	-
P. aeruginosa ATCC 27853 G(-)	625	625	625	625	625	>2500	>2500	-
K. pneumoniae ATCC 13883 G(-)	625	1250	625	625	625	1250	625	-
S. typhimurium ATCC 14028 G(-)	625	625	625	625	625	<19.5	156.3	-
E. hirae ATCC 9790 G(+)	625	625	625	312.5	312.5	19.5	156.3	-
P. vulgaris RSKK 96029 G(-)	1250	625	625	625	625	1250	1250	-
C. albicans ATCC 10231	625	312.5	1250	625	156.3	-	-	312.5
C. krusei ATCC 6258	625	156.3	156.3	156.3	156.3	-	-	<19.5
C. tropicalis Y-12968	312.5	312.5	1250	2500	<19.54	-	-	78.1
*Ampicillin (AMP), Chloramphenicol (C) (antibacterial) and Ketoconazole (K) (antifungal) were used as controls								

The MIC value of compound 28 against *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *P. vulgaris* RSKK 96029 was 625 µM. The effect of compound 28 on *E. coli* ATCC 35218 and *P. aeruginosa* ATCC 27853 was higher compared to that on *K. pneumoniae* ATCC 13883 and *P. vulgaris* RSKK 96029.

The MIC value of compound 29 against *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *P. vulgaris* RSKK 96029 was 625 µM. The effect of compound 29 on *E. coli* ATCC 35218 and *P. aeruginosa* ATCC 27853 was higher compared to that on *K. pneumoniae* ATCC 13883 and *P. vulgaris* RSKK 96029.

The MIC value of compound 30 against *E. coli* ATCC 35218 was 1250 µM; *S. aureus* ATCC 25923 was 78.1 µM; *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *P. vulgaris* RSKK 96029 were 625 µM; *C. albicans* ATCC 10231 was 156.3 µM, and *C. tropicalis* Y-12968 was <19.54 µM. The effect of compound 30 on *P. aeruginosa* ATCC 27853 and *C. tropicalis* Y-12968 was higher compared to that on *E. coli* ATCC 35218, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 13883, *P. vulgaris* RSKK 96029, and *C.*

albicans ATCC 10231. *P. aeruginosa* ATCC 27853 is the microorganism most affected by all compounds. Compound 30, which is effective against seven different microorganisms, is the most effective compound.

The MBC and MFC values of the compounds with respect to the microbes are shown in Table 4.2. For the evaluation of MBC/MFC, the same approach was taken according to what was previously explained for MIC value. The MBC/MFC values of compounds that demonstrate potential as bactericide/fungicide for the respective microbes are marked in yellow. The evaluation of a compound's potential was conducted by comparing its MBC/MFC value to that of the positive control. The positive control itself was selected from the highest concentration among the three predetermined substances (ampicillin, chloramphenicol, or ketoconazole) and its MBC/MFC value is marked in yellow. Positive control data was retrieved from (Kuzey et al., 2021).

Table 4.2. Minimum bactericidal and fungicidal concentrations of the controls and positive controls against test strains (MBC and MFC values are reported in µM)

MBC/MFC								
Compounds						Positive Controls		
Test Microorganisms	26	27	28	29	30	AMP	C	K
E. coli ATCC 35218 G(-)	2500	2500	2500	2500	2500	2500	>2500	-
E. coli ATCC 25922 G(-)	2500	1250	625	625	2500	<19.5	1250	-
B. cereus NRRL B-3711 G(-)	1250	>2500	>2500	312.5	>2500	2500	1250	-
B. subtilis ATCC 6633 G(+)	>2500	1250	>2500	1250	2500	19.5	78.1	-
S. aureus ATCC 25923 G(+)	2500	2500	2500	2500	156.3	<19.5	2500	-
E. faecalis ATCC 29212 G(+)	>2500	>2500	2500	2500	2500	312.5	625	-
P. aeruginosa ATCC 27853 G(-)	2500	1250	1250	1250	2500	2500	2500	-
K. pneumoniae ATCC 13883 G(-)	1250	2500	1250	1250	1250	1250	2500	-
S. typhimurium ATCC 14028 G(-)	2500	2500	2500	2500	2500	<19.5	1250	-
E. hirae ATCC 9790 G(+)	2500	>2500	2500	2500	2500	39.1	2500	-
P. vulgaris RSKK 96029 G(-)	1250	1250	1250	1250	625	>2500	2500	-
C. albicans ATCC 10231	2500	2500	2500	2500	2500	-	-	1250
C. krusei ATCC 6258	2500	2500	2500	2500	1250	-	-	156.3
C. tropicalis Y-12968	625	625	2500	2500	<19.54	-	-	1250
*Ampicillin (AMP), Chloramphenicol (C) (antibacterial) and Ketoconazole (K) (antifungal) were used as control								

The MBC value of compound 26 against *B. cereus* NRRL B-3711, *K. pneumoniae* ATCC 13883 and *P. vulgaris* RSKK 96029 has been determined to be 1250 µM. The MFC value of compound 26 against *C. tropicalis* Y-12968 has been determined to be 625 µM. The effect of compound 26 on *K. pneumoniae* ATCC 13883, *P. vulgaris* RSKK 96029, and *C. tropicalis* Y-12968 was equally high.

The MBC value of compound 27 against *P. aeruginosa* ATCC 27853 and *P. vulgaris* RSKK 96029 has been determined to be 1250 µM. The MFC value of compound 27 against *C. tropicalis* Y-12968 has been determined to be 625 µM. The effect of compound 27 on *P. aeruginosa* ATCC 27853, *P. vulgaris* RSKK 96029, and *C. tropicalis* Y-12968 was equally high.

The MBC value of compound 28 against *E. coli* ATCC 25922 has been determined at 625 µM, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *P. vulgaris* RSKK 96029 at 1250 µM. The effect of compound 28 on *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *P. vulgaris* RSKK 96029 was equally high.

The MBC value of compound 29 against *E. coli* ATCC 25922 has been determined at 625 µM, *B. cereus* NRRL B-3711 at 312.5 µM, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *P. vulgaris* RSKK 96029 at 1250 µM. The effect of compound 29 on *B. cereus* NRRL B-3711 was higher compared to *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *P. vulgaris* RSKK 96029.

The MBC value of compound 30 against *S. aureus* ATCC 25923 has been determined at 156.3 µM, *K. pneumoniae* ATCC 13883 at 1250 µM, and *P. vulgaris* RSKK 96029 at 625 µM. The MFC value of compound 30 against *C. tropicalis* Y-12968 was determined to be <19.54 µM. The effect of compound 30 on *C. tropicalis* Y-12968 was higher compared to *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 13883, and *P. vulgaris* RSKK 96029.

So, the bacteria *P. vulgaris* RSKK 96029 is the most affected by all compounds. The compound that has the most significant impact on microorganisms is compound 29 due to its effectiveness against five microorganisms. However, the compound that has a high ratio difference between positive control and microorganisms is compound 30.

Upon evaluating these results, it becomes apparent that there is no substantial difference between the concentrations of the compound that inhibit and completely eradicate the microorganisms.

4.2 Interaction of Phosphazene Compounds with DNA

4.2.1. The interaction of DNA and compounds

The interaction of compounds 26, 27, 28, 29, and 30 used in the study with plasmid DNA was investigated using agarose gel electrophoresis. The result is given in Image 4.2. The lane “PBR” refers to the control (untreated pBR322) whereas the consecutive lanes after it are of the mixture of plasmid DNA and the tested compounds at a decreasing concentration. The concentrations are as follows: (1): 5000 μM, (2): 2500 μM, (3): 1250 μM, (4): 625 μM, (5): 312.5 μM. Form I represents superhelix plasmid DNA, Form II represents nicked circular plasmid DNA, and Form III represents linear plasmid DNA.

The interaction of plasmid DNA with compound 26, carrying DASD substituents, decreases the mobility of DNA Form I and Form II as the concentration of the compound decreases. It also increases the intensity of DNA Form I.

The interaction of plasmid DNA with compound 27, carrying DASD substituents, decreases the mobility of DNA Form I and Form II as the concentration of the compound decreases. It also increases the intensity of DNA Form I. Additionally, Form III is also observed, indicating restriction of DNA.

The interaction of plasmid DNA with compound 28, carrying piperidine substituents, decreases the mobility of DNA Form I and Form II as the concentration of the compound decreases. It also increases the intensity of DNA Form II.

The interaction of plasmid DNA with compound 29, carrying piperidine substituents, decreases the mobility of DNA Form I and Form II as the concentration of the compound decreases. It also increases the intensity of DNA Form II.

The interaction of plasmid DNA with compound 30, carrying piperidine substituents, decreases the mobility of DNA Form I and Form II as the concentration of the compound decreases. It also increases the intensity of DNA Form II.

Image 4.2 shows that Form III was observed in the case where plasmid DNA was interacting with compound 27 in every variation of concentration. This implies that the restriction of DNA occurs due to the addition of the compound.

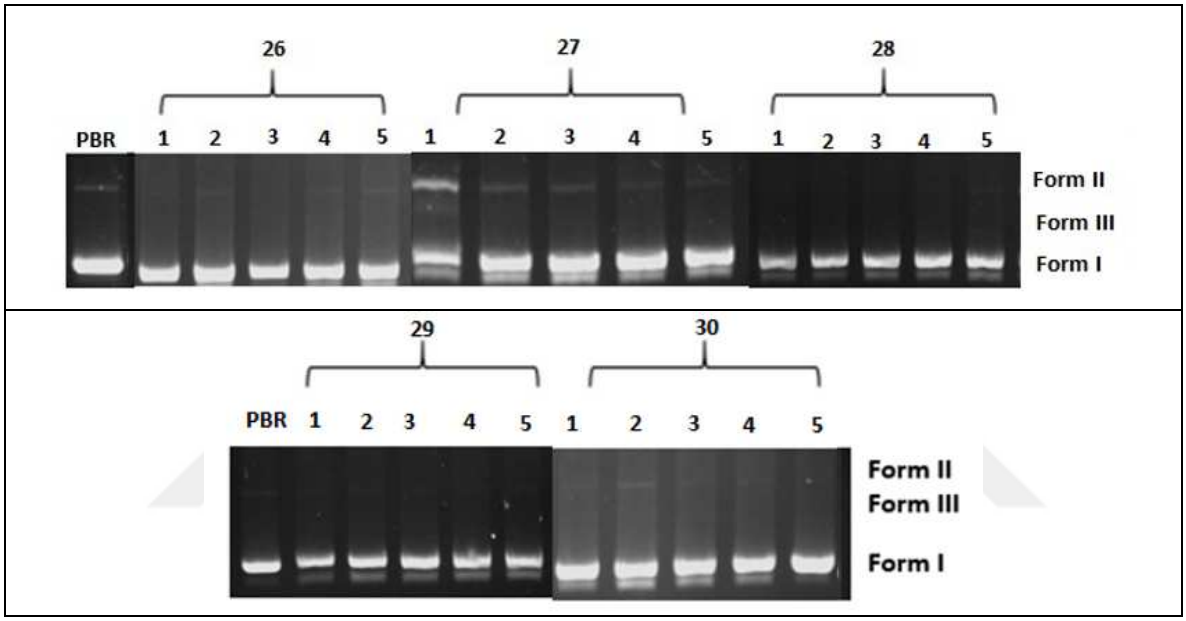


Image 4.2. Electrophoretograms indicating possible interaction between pBR322 plasmid DNA and compound 26 to 30 at a decreasing concentrations

4.2.2. Restriction enzyme digestion

To assess the binding affinity of compounds to nucleotides in DNA, plasmid DNA was subjected to enzymatic digestion at high concentrations 5000 μM, using the restriction endonucleases *Bam*HI and *Hind*III. The *Bam*HI enzyme recognizes the 5'-G/GATCC-3' region on DNA, while the *Hind*III enzyme recognizes the 5'-A/AGCTT-3' region on DNA. The DNA interacted with compounds 26, 27, 28, 29, and 30 were partly cleaved by the *Bam*HI enzyme. In addition DNA interacted with compounds 26, 27, 28, 29, and 30 were partly cleaved by *Hind*III enzyme. This suggests that compounds interact with DNA through nucleotides binding to A/A and G/G sites (Figure 4.3).

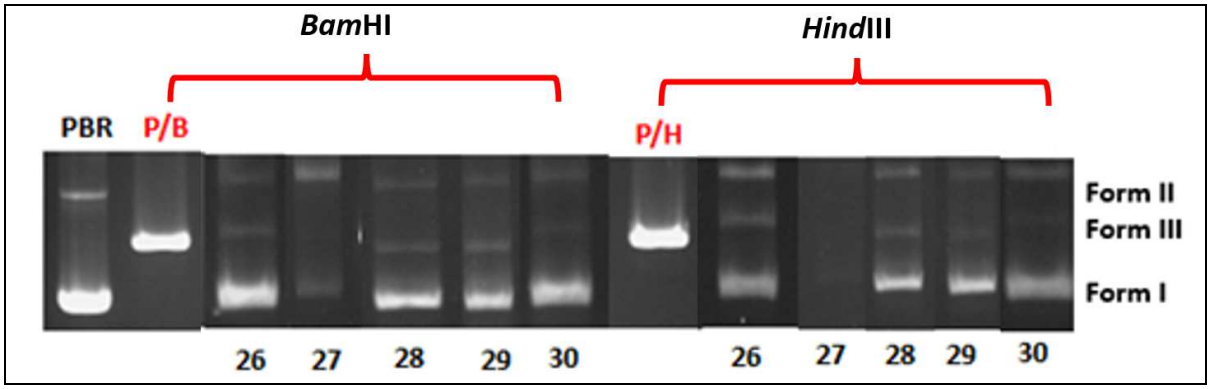


Image 4.3. Electrophoretograms of incubated mixtures of plasmid DNA subjected to digestion with *Bam*HI and *Hind*III where lane PBR, P/B, and P/H are untreated plasmid DNA

5. DISCUSSIONS

5.1. Efficacy of the Compounds

The compounds studied have been found to inhibit 7 out of the tested 14 microbes that were used in this study. The efficacy is retold using Table 5.1, which shows the ratio between the MIC of the chosen positive control for each microbe and the MIC of the studied compounds. Note that only compounds that produce cases where it is found to be effective were included in Table 5.1 (cases where the MIC ratio is higher than 1). In other words, if the compounds were found to require less concentration compared to the positive control to inhibit the microbes, then they are effective and are included in the Table 5.1.

Table 5.1. Ratio between MIC of positive control and compound for different compounds

MIC Ratio, Positive Control to Compound						
Compounds Name						
Test Microorganisms	26	27	28	29	30	Positive Controls
E. coli ATCC 35218 G(-)	2		4	4	2	AMP
S. aureus ATCC 25923 G(+)					2	C
P. aeruginosa ATCC 27853 G(-)	4	4	4	4	4	AMP and C
K. pneumoniae ATCC 13883 G(-)	2		2	2	2	AMP
P. vulgaris RSKK 96029 G(-)		2	2	2	2	AMP and C
C. albicans ATCC 10231					2	K
C. tropicalis Y-12968					4	K
*Ampicillin (AMP), Chloramphenicol (C) (antibacterial) and Ketoconazole (K) (antifungal) were used as controls						

Based on the MIC ratio of Table 5.1, *E. coli* ATCC 35218 is 2 times more susceptible to compounds 26 and 30 compared to ampicillin whereas it is 4 times more susceptible to compounds 28 and 29. *S. aureus* ATCC 25923 is 2 times more susceptible to compound 30 than chloramphenicol. *P. aeruginosa* ATCC 27853 is at least 4 times more susceptible to all of the tested compounds than both ampicillin and chloramphenicol. *K. pneumoniae* ATCC 13883 is 2 times more susceptible to compound 26, 28, 29, and 30 than ampecillin. *P. vulgaris* RSKK 96029 is 2 times more susceptible to compound 27, 28, 29, and 30 compared to both ampecillin and chloramphenicol. *C. albicans* ATCC 10231 is 2 times more susceptible to compound 30 compared to ketoconazole. *C. tropicalis* Y-12968 is at least 4 times more susceptible to compound 30 compared to ketoconazole.

Table 5.2. Ratio between MBC and MFC of positive control and compound for different compounds

MBC MFC Ratio, Positive Control to Compound						
Compounds Name						
Test Microorganisms	26	27	28	29	30	Positive Controls
E. coli ATCC 25922 G(-)			2	2		C
B. cereus NRRL B-3711 G(-)	2			8		AMP
S. aureus ATCC 25923 G(+)					16	C
P. aeruginosa ATCC 27853 G(-)		2	2	2		AMP and C
K. pneumoniae ATCC 13883 G(-)	2		2	2	2	C
P. vulgaris RSKK 96029 G(-)	2	2	2	2	4	AMP
C. albicans ATCC 10231						K
C. tropicalis Y-12968	2	2			64	K
*Ampicillin (AMP), Chloramphenicol (C) (antibacterial) and Ketoconazole (K) (antifungal) were used as controls						

Based on the MBC/MFC ratio of Table 5.2, *E. coli* ATCC 25922 is 2 times more susceptible to compounds 28 and 29 than chloramphenicol. *B. cereus* NRRL B-3711 is 2 times more susceptible to compound 26 than ampicillin whereas it is 8 times more susceptible to compound 29. *S. aureus* ATCC 25923 is 16 times more susceptible to compound 30 than chloramphenicol. *P. aeruginosa* ATCC 27853 is 2 times more susceptible to compounds 27, 28 and 29 than ampicillin and chloramphenicol *K. pneumoniae* ATCC 13883 is 2 times more susceptible to compounds 26, 28, 29, and 30 than chloramphenicol. *P. vulgaris* RSKK 96029 is 2 times more susceptible to compounds 26, 27, 28, and 29 compared to ampicillin whereas it is 4 times more susceptible to compound 30. *C. tropicalis* Y-12968 is 2 times more susceptible to compounds 26 and 27 compared to ketoconazole whereas it is 64 times more susceptible to compound 30.

5.2. Bacterial and Fungal Susceptibility

The monospirocyclotriphosphazene compounds used in this study consistently have pendant arms of (4-dimethylaminobenzyl)methyl with other pendant arms being either 1,4-dioxa-8-azaspiro[4.5]decane (DASD) or piperidinie. Antimicrobial activity was tested against 11 pathogenic bacteria and 3 pathogenic yeasts. The MIC results revealed that the bacterium *P. aeruginosa* ATCC 27853 was most affected by all compounds. This means that all fully substituted compounds with pendant arms of either 1,4-dioxa-8-azaspiro[4.5]decane

(DASD) or piperidine effectively combat *P. aeruginosa* ATCC 27853 as a pathogenic microorganism. Additionally, the tested compounds affected *E. coli* ATCC 35218, *K. pneumoniae* ATCC 13883 and *P. vulgaris* RSKK 96029, although not all compounds were affected. Overall, MIC results indicate that compounds 26-30 show the effectiveness in impeding pathogen growth at an average concentration of around 625 μM, while MBC results show effectiveness of the compound at killing the microorganism at an average concentration of around 1250 μM. The most effective compound at inhibiting the growth was compound 30 (piperidine pendant arms), proven against 7 types of microorganisms (*E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 13883, *P. vulgaris* RSKK 96029, *C. albicans* ATCC 10231, and *C. tropicalis* Y-12968).

When viewed based on MBC and MFC results, it is evident that *P. vulgaris* RSKK 96029 and *K. pneumoniae* ATCC 13883 are most affected by the monospirocyclotriphophazene in this study. All of the compounds have an impact on the various pathogenic microorganisms. However, compound 30 is the most effective compound at killing the microbes, even as much as 64 times higher vulnerability when considering the yeast, *C. tropicalis* Y-12968, compared to the positive control, ketoconazole.

The observation of the current result suggests that the compounds that have a piperidine functional group are more active than those with DASD functional group, demonstrating a higher antimicrobial effect on Gram-negative bacteria. This is especially true when observing compounds 30 and 27, where compound 30 showed relatively good efficacy across the board whereas compound 27 was only observed to be effective against 2-3 isolated cases of bacteria and fungi.

The result of this study favors compounds that has piperidine functional group (compound 28-30) as they have the potential to become an antimicrobial agent explicitly developed for the treatment of infections that are mainly caused by *P. aeruginosa* ATCC 27853 (-), *K. pneumoniae* ATCC 13883 (-), *P. vulgaris* RSKK 96029 (-), and *C. tropicalis* Y-12968. However, these compounds were to have worked againts *E. coli* ATCC 35218 (-), *E. coli* ATCC 25922 (-), *B. cereus* NRRL B-3711 (-), *S. aureus* ATCC 25923 (+), and *C. albicans* ATCC 10231 as well. The antimicrobial effect, both against bacteria, and fungi, of the compounds with a piperidine functional group has been reported before in the case of

halogenobenzene derivative compounds (Arslan et al., 2006). Their research pointed out that the position in which piperidine is substituted into the halogenobenzene derivative is also crucial in determining antimicrobial activity. Piperidine itself as an organic has been used as a building block and reagent in synthesizing various organic compounds that have medicinal benefits (Abdelshaheed et al., 2021). A developed compound with piperidine functional group was found to inhibit the synthesis of fatty acid in bacteria, effectively killing the bacteria (S.-S. Liu et al., 2023). Metal piperidine dithiocarbamate complexes have shown to have antimicrobial activity against *Staphylococcus aureus* and *Candida albicans* (Boadi et al., 2021). Thus, these studies, including the current work, suggest that peperidine-derived compounds or compounds with peperidine functional groups may have antimicrobial effects that are potentially significant to the development of a new antibiotic.

5.3. The Compounds in Relation with Cancer

Cancer is a global health problem responsible for one in six deaths worldwide. It is a highly complex set of disease conditions that develops gradually, leading to a general loss of growth control. However, recent advancements have drastically improved the understanding of the many pathways involved in cancer development and how to target them. New approaches, such as drugs and biological molecules, are now being employed for treatment. Since many drugs used in cancer treatment target DNA, the interactions between drugs and DNA, particularly in developing anticancer drugs, have gained significant attention.

The changes in DNA conformation resulting from the interaction of compounds with DNA can occur because DNA relies on the nature of binding or cleavage with DNA nucleobases (Akbaş et al., 2016). The technique used to assess the nature of DNA interaction with compounds is agarose gel electrophoresis using plasmid DNA (Elmas et al., 2018). The electrophoretic mobility that occurs on agarose gel is determined by the ability of each compound to unwind the supercoiled conformation of pBR322. Generally, agarose gel electrophoresis results in two basic bands for plasmids that are not treated with the test compounds (positive controls). The fastest migration occurs for the supercoiled form (SC) when circular plasmid DNA is subjected to gel electrophoresis. If shearing occurs in one strand, the supercoiled form will relax, producing a slower-moving open loop (OC). This happens due to the scission in one DNA strand. Linear DNA is produced from the supercoiled and open loop forms when both DNA strands are cleaved (Alaghaz and

Aldulmani, 2019). Form I is referred to as SC-DNA, form II as OC-DNA, and form III as L-DNA (Tanrikut et al., 2013). An analysis of phosphazene compounds (26, 27, 28, 29, and 30) used in this study interacting with plasmid DNA (pBR322) was conducted. It can be observed that all compounds cause different amounts of conformational changes and movement of plasmid DNA.

When the plasmid DNA pBR322 is incubated with the compound, bands of DNA related to supercoiled DNA, open circular, and linear forms are observed on both untreated and treated plasmid DNA. All examined compounds exhibit a decrease in the mobility of plasmid DNA. It has also been determined that compounds in this study cause cleavage in DNA as they can induce conformational changes in DNA. In fact, all DNA interacting with the compounds is partially cleaved by the *Bam*HI enzyme and partially cleaved by the *Hind*III enzyme. This indicates that the compounds interact with DNA through nucleotides binding to adenine-adenine (A/A) and guanine-guanine (G/G) sites. This interaction partially inhibits cleavage and digestion by the enzymes.

*Bam*HI and *Hind*III, as restriction enzymes, function by recognizing the sequences G/GATCC and A/AGCTT. These restriction enzymes can also cleave the phosphodiester bonds between adjacent nucleotide sites (Sambrook, 1989). *Bam*HI and *Hind*III restriction enzymes analyze the endonuclease restriction between compounds and the pBR322 plasmid DNA. These enzymes are also used to determine the affinity of compounds for G/G or A/A regions. The conversion of Form I and Form II to Form III results from the DNA cut by these restriction enzymes. *Bam*HI has only one binding site within the plasmid DNA. In previous research, phosphazene compounds have demonstrated varying effectiveness in their interactions with DNA (Tanrıkulu et al., 2019).

Over the years, studies on the biological activity of phosphazenes have revealed their potential as antimicrobial and anticancer agents. The biological effects of these phosphazenes vary based on the functional groups attached to the phosphazene compound. The phosphazene compounds used in this study exhibited different levels of antimicrobial and anticancer activity when interacting with DNA. At high concentrations (5000 μM), all compounds are only partly bound to DNA at the nucleotides in the enzyme cutting area.

5.4. Bacteriostatic and Bactericidal Drug

MIC signifies the minimum drug concentration at which bacterial growth is inhibited, while MBC, indicates the minimum drug concentration at which bacteria are being killed. Typically, there is a correlation between MBC and MIC, especially when considering two distinct classes of antibacterial drugs: bacteriostatic and bactericidal (Levison and Levison, 2009).

Bacteriostatic drugs are intentionally formulated to impede bacterial growth within the host. The mechanism by which bacteria inside the host are eliminated relies on the host's defense system. In instances where the host's defense mechanisms are insufficient in eradicating the infecting bacteria at the site of the infection, bacterial growth may resume after the bacteria have eliminated the drug. In such scenarios, bactericidal drugs are required to completely eliminate the bacteria (Levison and Levison, 2009). These two categories of pharmaceutical agents function differently, and their MIC and MBC values aid in distinguishing between the two of them.

Bacteriostatic drugs often exhibit MBC values that significantly differ from their MIC values, often being more than four times higher (Levison and Levison, 2009). It is crucial to note that the primary objective of these drugs is to kill bacteria indiscriminately. Therefore, the fact that the drug only impedes the growth and does not eliminate the bacteria should not be perceived as a disadvantage. Examples of primarily bacteriostatic drugs include tetracyclines, macrolides, and chloramphenicol (Loree and Lappin, 2023). In contrast, bactericidal drugs tend to have MBC values equal to or, at most, four times higher than their MIC values (Levison and Levison, 2009). Examples of such drugs include quinolones and ampicillin (Bernatová et al., 2013; Peechakara and Gupta, 2024; von Rosenstiel and Adam, 1994).

Table 5.3 shows the MIC and MBC value of the tested compounds for the different bacteria species in the current study. Chloramphenicol, which is classified as a bacteriostatic drug (Loree and Lappin, 2023), is given a green shade. The behaviour of chloramphenicol in response to various of the tested bacteria may differ in a the sense that both the MBC and MIC change with the change of the bacteria species. For a bacteriostatic drug. MBC value usually would be more than four times higher than its MIC (Levison and Levison, 2009). In

such case, the shade of green is lighter. When MBC is less than four times the MIC, then the shade of green will be darker. As expected, the MBC values of chloramphenicol are mainly more than four times the MIC, with 7 out of the 11 tested bacteria showing such cases. This observation asserts the fact that chloramphenicol is a primarily bacteriostatic drug.

In the case of ampicillin, in Table 5.3, tan shade was given to distinguish it from chloramphenicol. In contrast to chloramphenicol, a bacteriostatic drug, ampicillin is a bacteriocidal drug (Peechakara and Gupta, 2024). This can be seen by how the MBC is either the same as to its MIC or at least not more than fourfold of it (darker tan shade). From 11 of the tested bacteria, only two of them have MBCs that are more than four times their MIC (lighter shade of tan).

Based on the analysis of Table 5.3, the tested phosphazene compound in this study have seen behaviour similar to ampicillin than that of chloramphenicol. This statement is supported by how for most of the cases, compound 26-30's MBC is equal to or less than four times its MIC (darker shade of tan). Whereas in certain cases the MBC value is more than four times the MIC (lighter shade of tan). Thus, it can be deduced that phosphazene compounds that are tested in the current study are mainly candidates for bactericidal drugs more so than bacteriostatic drugs.

Table 5.3. MIC and MBC of compound 26-30 (bacteriostatic/bacteriocidal drug)

Compounds							Positive Controls	
Test Microorganisms		26	27	28	29	30	AMP	C
E. coli ATCC 35218 G(-)	MIC	1250	2500	625	625	1250	2500	625
	MBC	2500	2500	2500	2500	2500	2500	>2500
E. coli ATCC 25922 G(-)	MIC	2500	625	312.5	625	2500	<19.5	78.1
	MBC	2500	1250	625	625	2500	<19.5	1250
B. cereus NRRL B-3711 G(-)	MIC	625	>2500	625	312.5	1250	156.3	156.3
	MBC	1250	>2500	>2500	312.5	>2500	2500	1250
B. subtilis ATCC 6633 G(+)	MIC	>2500	1250	>2500	1250	1250	<19.5	78.1
	MBC	>2500	1250	>2500	1250	2500	19.5	78.1
S. aureus ATCC 25923 G(+)	MIC	1250	1250	1250	625	78.1	<19.5	156.3
	MBC	2500	2500	2500	2500	156.3	<19.5	2500
E. faecalis ATCC 29212 G(+)	MIC	625	625	625	625	625	<19.5	312.5
	MBC	>2500	>2500	2500	2500	2500	312.5	625
P. aeruginosa ATCC 27853 G(-)	MIC	625	625	625	625	625	>2500	>2500
	MBC	2500	1250	1250	1250	2500	2500	2500
K. pneumoniae ATCC 13883 G(-)	MIC	625	1250	625	625	625	1250	625
	MBC	1250	2500	1250	1250	1250	1250	2500
S. typhimurium ATCC 14028 G(-)	MIC	625	625	625	625	625	<19.5	156.3
	MBC	2500	2500	2500	2500	2500	<19.5	1250
E. hirae ATCC 9790 G(+)	MIC	625	625	625	312.5	312.5	19.5	156.3
	MBC	2500	>2500	2500	2500	2500	39.1	2500
P. vulgaris RSKK 96029 G(-)	MIC	1250	625	625	625	625	1250	1250
	MBC	1250	1250	1250	1250	625	>2500	2500

Nomenclature:

	or	MBC more than 4 times MIC (bacteriostatic drug)
	or	MBC is 4 times MIC or less (bactericidal drug)
↑ Primarily Bacteriocidal		↑ Primarily Bacteriostatic

6. CONCLUSION AND RECOMMENDATIONS

The results and recommendations derived from the research conducted within the framework of this master's thesis are outlined below:

1. This study focused on monospirocyclotriphosphazene derivatives (compound 26-30). Piperidine and 1,4-dioxa-8-azaspiro[4.5]decane (DASD) were two functional groups substituted into the compounds' pendant arms.
2. Monospirocyclotriphosphzane compounds in this study exhibited more effective antimicrobial activity against microorganisms when they had a piperidine functional group instead of DASD. All compounds demonstrated a higher effectiveness observed against Gram-negative bacteria than Gram-positive ones. The MIC, MBC, and MFC values of compounds with notable antimicrobial activity were determined to inhibit or kill microorganisms at low concentrations ranging from (<19.54) to (>2500 μM). The findings suggest the potential use of these compounds as antimicrobial agents, emphasizing the need for further research to elucidate the mechanisms underlying their antimicrobial effects on microorganisms.
3. The interaction of the compounds with DNA was assessed using the agarose gel electrophoresis method. It was observed that most compounds caused the fragmentation of closed circular DNA, forming a linear band III. Additionally, the compounds were found to interact with DNA, leading to a reduction in its mobility. This implies that these compounds may bind to DNA through non-covalent electrostatic interactions. Enzymatic digestion with *Bam*HI and *Hind*III revealed that some compounds bound to specific regions of DNA. It is recommended to investigate whether compounds not binding to DNA in these regions interact with different DNA regions by employing various restriction enzymes.
4. Based on the results of all biological activity experiments explored in this thesis, it is evident that compounds containing piperidine substituents exhibit higher activity.

In summary, the research suggests that phosphazene derivatives with piperidine side groups have the potential to serve as effective antimicrobial and anticancer agents. Further development and consideration of other biological activities in phosphazene compounds could contribute to novel research in this field.

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Gazili olmak ayrıcalıktır