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Contents

ABDİİBRAHİM

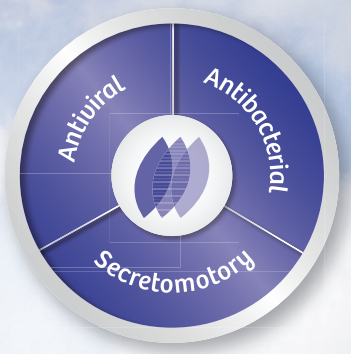
Respiratory Track Infections

First choice:[1,4]

Umca®

The best researched physomedicine in the treatment of RTIs

Unique Triple Mode of Action[3]



- **Reduces the severity of the symptoms and shortens the duration of disease[1,2]**
- **Excellent safety and tolerability profile[1]**
- **Suitable for the whole family[1]**

References:
1. Ligogup V, et al. Explore (NY) 2007;3(6):69-73 2. Bachert C, et al. Rhinology 2009:47:51-58 3. Michaelis, et al. Phytomedicine 2011; 18: 384-386 4. UMCA prospectus

UMCA SOLUSYON BİLEŞİMİ: Her 100 g çözelti etkin madde olarak 80 g Pelargonium sidoides kökü sıvı ekstresi, çözücü ve koruyucu olarak etanol ve gliserol içermektedir, TIBBİ ÖZELLİKLERİ: Umca Pelargonium sidoides'in kökünden elde edilen bir öz içermektedir, Pelargonium sidoides'ten elde edilen özün bronşit, sinüzit, anjin (boğaz ağrısı), viral enfeksiyonlara bağlı burun akıntısı ve farenjit olgularında etkili olduğu saptanmıştır. Aynı zamanda bağışıklık sistemini güçlendirici antiviral özelliklere sahiptir, Ayrıca bazı bakterilere karşı antibakteriyel etkisinin yanısıra antioksidan özelliklerine sahiptir, Burun dışında, organizmanın bağışıklık sistemini güçlendirdiği ve solunum yolu mukozasındaki titrek tüylerin vurum sıklığını artırarak balgam söktürücü etkiye sahip olduğu da bildirilmiştir, Bu nedenle Umca, değişik akut ve kronik enfeksiyonların özellikle de üst solunum yolları enfeksiyonları ve kulak-burun-boğaz enfeksiyonlarının tedavisi için uygundur, Umca uygulaması ile öksürük, ateş, boğaz ağrısı, halsizlik-yorgunluk gibi yakınmalarda hızlı bir iyileşme sağlanabilmektedir, ÖNERİLEN KULLANIM YERİ: Umca, akut ve kronik enfeksiyonlar, özellikle de solunum yolları enfeksiyonları (örneğin soğuk algınlığı ve bronşit gibi) ve kulak-burun-boğaz enfeksiyonları (örneğin sinüzit, anjin, rinofarenjit gibi) tedavisine yardımcıdır, Umca öksürük, ateş, boğaz ağrısı, halsizlik-yorgunluk gibi yakınmaların tedavisine yardımcıdır, KULLANILMAMASI GEREKEN DURUMLAR: Umca'nın içinde bulunan maddelerden herhangi birine karşı aşırı hassasiyeti olanlarda kullanılmamalıdır, Kanama eğiliminin yüksek olduğu hastalarda veya kan pıhtılaşması önleyen ilaçlar alanlarda kullanılmamalıdır, Yeterli klinik deneyim olmadığı için, ağır karaciğer ve böbrek rahatsızlıkları olanlarda kullanılmamalıdır, UYARILAR/ÖNLEMLER: Umca 1 yaşın altındaki bebeklerde önerilmemektedir, Gebeler veya emziren anneler tarafından kullanımı önerilmemektedir, Etanol içermesi nedeniyle araç ve makine kullanımında dikkatli olunmalıdır, YAN ETKİLER/ADVERS ETKİLER: Enfeksiyon durumlarında örneğin akut bronşitte karın ağrısı, mide yanması, bulantı ve ishal gibi yakınmalar görülebilir, Nadiren, bu yakınmalar Umca kullanımına bağlı olabilir, Nadir vakalarda, hafif diş eti veya burun kanaması görülebilir, Umca'nın içinde bulunan maddelere karşı aşırı hassasiyeti olanlarda çok nadiren aşırı duyarlılık reaksiyonlar gelişebilir, Bu tür reaksiyonlarda yüzde ödem (şişlik), nefes darlığı ve kan basıncında düşüş görülebilir ve ürünün ilk alımından sonra gelişebilir, Böyle bir durumda derhal doktora başvurulmalıdır, İstenmeyen bir etki görüldüğü zaman Sağlık Bakanlığı Türkiye Farmakovijilans Merkezi (TÜFAM)'ne bildiriniz, İLAÇ ETKİLEŞİMLERİ VE DİĞER ETKİLEŞİMLER: Kumarin türevleri ile birlikte kullanılması durumunda, kan pıhtılaşmasını engelleyici etkide bir artış meydana gelebilir, Bu nedenle koagülasyonu inhibe eden ilaçlar ile birlikte kullanılmamalıdır, GÜNLÜK KULLANIM ŞEKLİ VE DOZU: Yetişkinler ve 12 yaş üzeri çocuklarda günde 3 defa 30 damla, 6-12 yaş arası çocuklarda günde 3 defa 20 damla, ve 1-5 yaş arası çocuklarda günde 3 defa 10 damla şeklinde kullanılır, Damlalar, yemeklerden 30 dakika önce bir miktar sıvı ile birlikte içilmelidir, Hastalığın nüksetmemesi için, hastalığın belirtileri hafiflemesini takiben ilacın kullanımına birkaç gün daha devam edilmesi önerilir, Umca şişesi açıldıktan sonra oda ısında muhafaza edildiği takdirde 6 ay boyunca kullanılabilir, TİCARİ TAKDİM ŞEKLİ: 20 ve 50 ml'lik kendinden damlalıklı cam şişelerde, İZİN SAHİBİ: Abdi İbrahim İlaç San, ve Tic, A.Ş, Reşitpaşa Mah, Eski Büyükdere Cad, No:4 34467 Maslak / Sarıyer / İSTANBUL İZİN TARİH VE NUMARASI: 11.03.2008-2008/10 ÜRETİM YERİ & LİSANS SAHİBİ: Dr. Willmar Schwabe GmbH & Co. KG Willmar-Schwabe Straße 4, 76227, Karlsruhe / ALMANYA PAREKENDE SATIŞ FİYATI: 20 mL solusyon 35 TL, 50 mL solusyon 59 TL "25°C'nin altındaki oda sıcaklığında saklayınız," "Çocukların göremeyeceği, erişemeyeceği yerlerde ve ambalajında saklayınız," BU ÜRÜNÜN TIBBİ YARARI GELENEKSEL KULLANIMA VE LİTERATÜRE DAYANMAKTADIR. TIBBİ MÜSTAHZAR (İLAÇ) OLARAK DEĞERLENDİRİLMEMİŞTİR. SADECE ECZANELERDE SATILIR.

Aims and Scope of Acta Pharmaceutica Sciencia

Acta Pharmaceutica Sciencia is a continuation of the former "Eczacılık Bülteni" which was first published in 1953 by Prof. Dr. Kasım Cemal GÜVEN's editorship. At that time, "Eczacılık Bülteni" hosted scientific papers from School of Medicine-Pharmacy Branch, Istanbul University, Turkey.

Starting from 1984, the name of the journal was changed to "Acta Pharmaceutica Turcica" and became a journal for national and international manuscripts, in all fields of the pharmaceutical sciences in both English and Turkish. (1984-1995, edited by Prof. Dr. Kasım Cemal GÜVEN, 1995-2001, edited by Prof. Dr. Erden GÜLER, 2002-2011, edited by Prof. Dr. Kasım Cemal GÜVEN)

Since 2006, the journal has been publishing only in English with the name, "Acta Pharmaceutica Sciencia" that represents internationally excepted high level scientific standards.

The journal has been publishing quarterly per year except an interval from 2002 to 2009 which released its issues trimestral in a year. Publication was discontinued from the end of 2011.

With this issue in 2017, Acta Pharmaceutica Sciencia is continuing publication with the reestablished Editorial Board and also with support of you as precious scientists.

Yours Faithfully

Prof. Dr. Şeref DEMİRAYAK
Editor

ABDİİBRAHİM

Big Trouble of Small Teeth

Apply it confidently, let him always laugh...

"#1 Teething Gel in Turkey[1]"

Reference: 1. IMS 2016

DENTINOX® DİŞ JELİ BİLEŞİMİ: Beher gram jel, etkin madde olarak Lidokain hidroklorür 3.4 mg, Hidroksipolietoksidodekan-600 3.2 mg, Kamomil tentürü 150 mg, yardımcı madde olarak Ksilitol 90.0 mg, %70'lik Sorbitol solüsyonu 100.0 mg, Disodyum edetat 1.0 mg, Propilen glikol 150.0 mg, Sodyum hidroksit 4.625 mg, Sodyum sakarin 2.0 mg içerir. **FARMAKOLOJİK:** DENTINOX®'un bileşiminde bulunan Lidokain ve Hidroksipolietoksidodekan lokal anestezik etkilerinden ötürü bebek ve küçük çocukların diş çıkarma döneminde görülen ağrıyı dindirir ve gingiva stomatitisi ağrısını engeller. Kamomil tentürü ise bileşimindeki maddeler nedeni ile bukkal mukozanın enflamasyonunu giderir. **ENDİKASYONLARI:** DENTINOX®, çocuklarda diş çıkarma veya Gingiva stomatitisi sırasında görülen ağrılı ve enflamasyonlu durumların giderilmesinde kullanılır. **KULLANILMAMASI GEREKEN DURUMLAR:** Etkin madde ya da bileşimindeki maddelerden herhangi birine karşı aşırı duyarlılığı olan hastalarda kontrendikedir. **UYARILAR/ÖNLEMLER:** DENTINOX® sorbitol solüsyonu içerdiğinden nadir kalıtımsal fruktoz intolerans problemi olan hastaların bu ilacı kullanmamaları gerekir. DENTINOX® propilen glikol içerdiğinden irritasyona neden olabilir. DENTINOX® sodyum içermektedir. Bu durum kontrollü sodyum diyetinde olan hastalar için göz önünde bulundurulmalıdır. Ancak kullanım yolu nedeniyle herhangi bir uyarı gerekmemektedir. **YAN ETKİLER/ADVERS ETKİLER:** Bugüne kadar bildirilen bir yan etkisi mevcut değildir. Etkin ya da yardımcı maddelerden herhangi birine duyarlılığı olan kişilerde advers etki görülebilir. İstenmeyen bir etki görüldüğü zaman Sağlık Bakanlığı Türkiye Farmakovijilans Merkezi (TÜFAM)'ne bildiriniz. **İLAÇ ETKİLEŞİMLERİ VE DİĞER ETKİLEŞİMLER:** Bildirilmemiştir. **GÜNLÜK KULLANIM ŞEKLİ VE DOZU:** Hekim tarafından başka bir öneri olmadıkça; günde 2-3 kez DENTINOX® uygulaması yeterlidir, diş etinin etkilenmiş bölgesine temiz bir parmak ya da pamuk yardımı ile uygulanıp hafifçe masaj yapılır. Özellikle yemeklerden sonra ve uykudan önce uygulanmalıdır. **TİCARİ TAKDİM ŞEKLİ:** 20 g'lık Alüminyum tüp. **İZİN TARİH VE NUMARASI:** İlk ruhsat tarihi: 20.04.1993, Ruhsat yenileme tarihi:15.12.2010, ruhsat no, 164/20 **ÜRETİM YERİ & LİSANS SAHİBİ:** DENTINOX® GESELLSCHAFT (Almanya) lisansı ile, Abdi İbrahim İlaç San. ve Tic. A.Ş. Reşitpaşa Mah. Eski Büyükdere Cad. No: 4 34467 Maslak/Sarıyer/İSTANBUL **PERAKENDE SATIŞ FİYATI:** 10,63TL (20.02.2017). "25°C'nin altındaki oda sıcaklığında saklayınız."

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Bacteriological Analysis of Indoor Air of Three Hospitals in Lahore, Pakistan

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[2]Hacettepe University, Faculty of Pharmacy, Department of Pharmacology, 06100 Sıhhiye, Ankara, Turkey.

ABSTRACT

The aim of present study was to determine the quality of indoor air of different wards of three hospitals in Lahore to check the atmosphere of these hospitals might be a potential source of hospital acquired infections. Using Buck's biological air sampler, microbiological samples were collected from different units of three hospitals. The average amount of microbial loads was determined by using colony counter (Suntex, CC-570). The present study showed that bacterial population was increased in Out Patient Department (75.89%), Intensive Care Unit (78.43%) and Nursery Unit (81.5%) statistically significant in three hospitals at evening time. The bacteria isolates were *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli*. It was found that indoor air quality of the three hospitals varied depending on disinfection process frequency and number of individuals present in environment. The average number of microorganisms in the atmosphere of hospitals did not fulfill the maximum allowed colony forming units per cubic meter.

Keywords: Hospital acquired infection; indoor air; microbial load

INTRODUCTION

One of the primary goal of a hospital is control of infection spread. However sometimes when a patient goes to hospital for the treatment of some other condition, he acquires an infection during his stay at hospital, referred as hospital acquired infection (HAI). HAI is also known as nosocomial infection. A great attention has been focused on HAIs and their control strategies because of increase number of nosocomial infections over the past decade.[1] The result of HAI may range from longer hospital stays to development of a condition that needs sur-

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gery or even to death.[2] Hence, HAIs elevate morbidity, mortality and treatment costs.[3] HAIs occurrence takes place worldwide and affecting the population of developing as well as developed countries.[4] According to a study conducted by World Health Organization (WHO), in 55 hospitals of 14 countries representing four WHO regions (Europe, Eastern Mediterranean, South-East Asia and Western Pacific) an average of 8.7% of hospital patients had acquired nosocomial infection. Another study showed that there was 9.1% (Greece), 7% (Spain), 5.1% (Norway) and 4.6% (Slovenia) annually HAIs prevalence.[5–7]

Various factors including air, surfaces, hands, water and a number of routes such as oral, intravenous or surgery may be involved in spread of HAIs. Indoor patients are more prone to HAIs because of their underlying disease conditions.[8] Transmission of microorganisms that cause HAIs usually takes place through hands of physicians, nurses and other paramedical staff in intensive care unit (ICU).[9] Viruses and fungi can cause HAIs but bacteria are the main causative agents involved in nosocomial infections.[10] The bacteria that are most commonly involved in HAIs include *Streptococcus spp., Acinetobacter spp., Enterococci, Pseudomonas aeruginosa (P. aeruginosa*), coagulase-negative *Staphylococci, Staphylococcus aureus, Bacillus cereus (B. cereus),* Legionella and Enterobacteriaceae family members including *Proteus mirabilis, Klebsiella pneumonia, Escherichia coli (E. coli), Serratia marcescens*. Out of Enterococci, *P. aeruginosa*, *S. aureus* and *E. coli* have a major role.[11]

HAIs and types of causative agents may vary with in different sections of the same hospital or from hospital to hospital.[4] Generally, nursery department has more frequent and more severe HAIs as newborn infants are more susceptible to infections and another cause may be their prolong hospital stay.[12] Outpatient department (OPD) may also be a source of HAIs especially for the patients undergoing intravenous treatment.[13]

HAIs are becoming increasingly more important in Pakistan. Therefore, the objectives of present study was to determine the average amount of microorganism loads in the atmosphere of three hospitals at Lahore, Pakistan in order to show that whether the atmosphere of these hospitals might be a potential source of HAIs caused by various microbial pathogens.

METHODOLOGY

Sample Location

One hundred and sixty two air samples were collected from outpatient department, intensive care unit and nursery sections of hospital 1, hospital 2 and hospital 3 in Lahore, Pakistan.

Media Preparation

According to manufacturer's instructions, tryptic soy agar (TSA) media were prepared. Prepared media was autoclaved at 125°C for 15 minutes. After autoclaving, placed it in water bath at 45°C. When its temperature reached at 45°C, transferred the media into petri dishes under laminar flow hood (LFH). Before the preparation of media plates, transferred the media bottles and petri dishes in class "A" area under LFH for 15 min. 15-20 ml media had been poured into each petri dish and allowed to solidify. After 15 min removed the plates from LFH and incubated them in incubator for 24 hours at 35°C. Next day, if there was any growth on media plates, those were discarded and not used for research's purposes.

Air Sampling

Buck's biological air sampler was used for air sampling. Biological air sampler was sterilized at 180°C for 48 hours in hot air oven. Wrapped with sterilized aluminum paper biological air sampler and petri dishes were carried to the sampling places. Placed the petri dish in cavity of air sampler, closed its perforated lid, pressed ON button, and adjusted the air suction time for 10 min (100L/mint). After 10 min placed the glass lid on petri dish and wrapped it again with sterilized aluminum foil. For second air sampling, the stainless steel perforated lid was mopped with 70% isopropyl alcohol and placed on flame for sterilization purpose. Repeated the same procedure. This procedure was performed for taking air sample at 9 am and 5 pm for 3 times from June 2013 to August 2013, once in every month. Samples were carried to Microbiology section of Medisave Pharmaceuticals, Lahore where analysis was done. After that, petri dishes were incubated in hot air incubator at 32-35°C for 24 hours. Control petri dishes were not exposed to air but were placed there for 10 min and same procedure was performed. On next day, plates were studied for colony forming unit count under colony counter (Suntex, CC-570) and a number of tests (Gram Staining test, Eosin methylene blue agar test for *E. coli*, Mannitol salt agar test for *S. aureus*, Catalase test for *E. coli*, Klebsiella and *S. aureus* , IMVIC test for *E. coli* and Klebsiella, Vogues-Proskauer test for *E. coli* and Klebsiella, Simmons's citrate test for *E. coli* and Coagulase test for *S. aureus*) were performed to isolate and identify microorganisms.[14] Colony forming units per cubic meter (cfu/m^3) were calculated by using following formula;

$$\frac{\text{Colony count in the petri dish (cfu)}\times 1000}{\text{Aspirated air (L)}} = \text{cfu/m}^3$$

Statistical Analysis

Data was analyzed by mean, standard deviation and standard error of the mean. Statistical analysis was done by Student's t test and $p<0.05$ was accepted as significant. Graph Pad Prism 5 was used for analysis.

RESULTS

Out Patient Department

Our studies showed that number of colonies of microorganisms at evening time had been increased significantly as compared to those of morning time in OPD of all three hospitals. Control group had zero number of colonies and taken as reference.

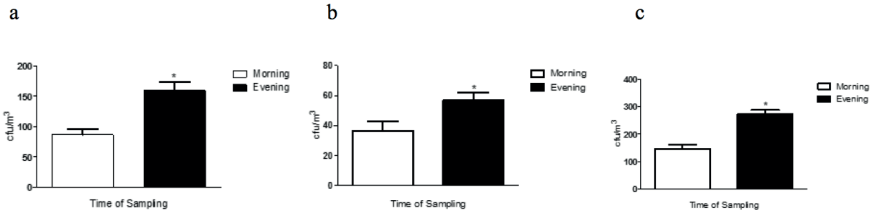

Figure 1. The number of colonies forming units per cubic meter (cfu/m3) at morning at OPD department of (a) hospital 1 (86.78 ± 9.131), (b) hospital 2 (36.78 ± 6.112) and (c) hospital 3 (146.8 ± 13.93). At evening hospital 1 (159.5 ± 13.99), (b) hospital 2 (56.78 ± 5.408) and (c) hospital 3 (273.8 ± 14.72). (*$p<0.05$ compared to morning group; n=9). (Morning at 9am, Evening at 5pm).

Intensive Care Unit Department

It had been found that no. of colonies increased at evening time samples as compared to those which were exposed at morning soon after disinfection process in ICU department of three hospitals. Unexposed petri dishes had been taken as reference having no single colony.

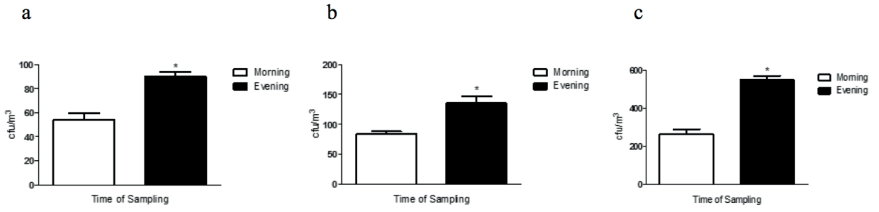

Figure 2. The number of colonies forming units per cubic meter (cfu/m3) at morning at ICU department of (a) hospital 1 (54.44 ± 4.894), (b) hospital 2 (84.11 ± 3.557) and (c) hospital 3 (262.0 ± 27.49). At evening hospital 1 (90.11 ± 4.050), (b) hospital 2 (135.1 ± 12.05) and (c) hospital 3 (548.6 ± 20.78). (*$p<0.05$ compared to morning group; n=9). (Morning at 9am, Evening at 5pm).

Nursery Department

Petri dishes exposed for air sampling at evening time at nursery department have increased number of colonies formation when compared with petri dishes exposed at morning time at hospitals. Control unexposed petri dishes had no formation of colonies after 24 hour incubation.

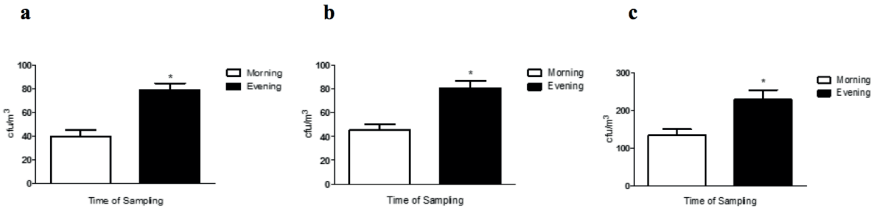

Figure 3. The number of colonies forming units per cubic meter (cfu/m3) at morning at nursery department of (a) hospital 1 (39.89 ± 5.561), (b) hospital 2 (45.44 ± 4.790) and (c) hospital 3 (134.8 ± 15.02). At evening (a) hospital 1 (78.89 ± 5.675), (b) hospital 2 (80.67 ± 6.245) and (c) hospital 3 (228.1 ± 25.86). (*p<0.05 compared to morning group; n=9). (Morning at 9am, Evening at 5pm).

Table 1. Number of live microorganisms in air samples collected from OPD at morning and evening in Hospital 1, Hospital 2 and Hospital 3 (n=3).

Samples	Hospital 1				Hospital 2				Hospital 3			
	Morning		Evening		Morning		Evening		Morning		Evening	
	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD
1st month	86.66	26.1	182	25.23	54.33	16	67	23	168.33	44.37	260.33	46.37
2nd month	80.66	17.5	160	53.67	35.33	11.93	56.66	56.66	130.66	43	298.33	7.63
3rd month	69	25	140	26.11	20.66	9.5	46.66	8	141.33	45	269.66	59.18
Avg	78.77	22.86	161	35	36.77	12.47	56.77	29.22	146.77	44.12	276.10	37.7

Avg: Average; SD: Standart Deviation

Table 2. Number of live microorganisms in air samples collected from ICU at morning and evening in hospital 1, hospital 2 and hospital 3 (n=3).

Samples	Hospital 1				Hospital 2				Hospital 3			
	Morning		Evening		Morning		Evening		Morning		Evening	
	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD
1st month	48.66	15.63	83	13.22	86	8.71	154	45.73	255	48.28	558.33	101.3
2nd month	62.66	18	92.66	7.37	83.33	11.59	113.33	10.69	273	147.23	522	55.5
3rd month	52	11.53	94.6	15.63	83	15.39	138	42	258	54	565.33	24.17
Avg	54.44	15.05	90.10	12.07	84.11	11.89	135.11	32.8	262	83.17	548.53	60.32

Avg: Average; SD: Standart Deviation

Table 3. Number of live microorganisms in air samples collected from nursery section at morning and evening in hospital 1, hospital 2 and hospital 3 (n=3).

Samples	Hospital 1				Hospital 2				Hospital 3			
	Morning		Evening		Morning		Evening		Morning		Evening	
	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD	Avg (cfu/m^3)	SD
1st month	38	24.63	84.66	13.86	48	20.22	63.66	14.57	152.33	42.89	283.66	62.93
2nd month	32	12.76	65	10.58	36.66	11.5	95	13.22	106.33	24.7	211.33	20.74
3rd month	49.66	10	87	20.42	51.66	10	83.33	16.25	145.66	61.77	186.33	45.5
Avg	39.88	15.79	78.88	14.95	45.44	13.9	80.66	14.68	134.77	43.12	227.1	43.1

Avg: Average; SD: Standart Deviation

DISCUSSION

Nosocomial infections have become known as one of the most important health issue nowadays. Nosocomial infections lead to increase human suffering and increase treatment cost.[4] One study showed that death rate associated with HAIs has been elevated to several of top ten leading causes of death in U.S.[15] It had been reported that HAI rate in pediatric oncology department of Aga Khan hospital, Karachi, Pakistan was 3.1 per 100 hospitalized patients.[16] In Liaqat University hospital, Pakistan, the frequency of nosocomial infection was 29.13% in intensive care unit.[17] These findings force us to redesign and improve preventive measures.

Microbial quality of the indoor air of different departments of hospital is one of the important factor related to nosocomial infections.[18] Dust arising from human movements such as sweeping or bed making in the hospitals may be a major offender of air borne contamination.[19] Exposure of hospitalized patients to these air borne microorganisms often leads to infections, respiratory problems and allergic reactions.[20] As the hospital environment is a source of acquired infections, knowledge of microbial flora of indoor air of various units of hospitals becomes mandatory to find out the possible causes of infections.

The present study aimed to get knowledge about the quality of indoor air of different departments of three hospitals. Number of microorganisms was counted, culture was studied to isolate the causative agents and a number of chemical tests were performed to confirm the bacterial species. In this study, only bacterial data was studied as bacteria play major role in spreading HAIs.[10] For the selection criteria of hospital sections, it was kept in mind that one general ward (OPD) and two sensitive areas (ICU and nursery section) to check atmosphere microbial contamination. Sampling time was once early at morning just after disinfection process and the second time was at evening when sections were ex-

posed a large number of individuals. So importance of air disinfection process was also evaluated.

It was found that the number of air borne microorganisms was significantly elevated in evening time air samples of three departments OPD (75.89%), ICU (78.43%) and nursery unit (81.5%) in all three hospitals as shown in figure 1, 2 and 3 respectively .The number of microorganisms was less at morning time samples just air disinfection process. These findings showed the effectiveness of disinfection process in all three hospitals. However, the elevated number of colony forming units per cubic meter (cfu/m^3) at evening time was due to some factors including the number of patients and visitors at wards during whole day.[21]

The frequency of microorganisms at morning/evening in OPD section of hospital 1 (86.78 ± 9.13/159.5 ± 13.99), hospital 2 (36.78 ± 6.11/56.78 ± 5.41) and hospital 3 (146.8 ± 13.93/273.8 ± 14.72) fulfilled the criteria 300-400 cfu/m^3 for patients wards. In ICU, number of microorganisms at morning time of hospital 1 (54.44 ± 4.89) met the standard criteria but hospital 2 (84.11 ± 3.55) and hospital 3 (262.0 ± 27.49) did not, while at evening time sampled air for hospital 1(90.11 ± 4.05), hospital 2 (135.1 ± 12.05) and hospital 3 (548.6 ± 20.78) could not confirm to permitted range of microbes. As nursery sections are also considered as first-class environments according to DIN 1946/4 standards, allowed number of microbes for nursery is <70 CFU/m^3 as in case of ICU. The number of microorganisms in hospital 1(39.89 ± 5.561) and hospital 2 (45.44 ± 4.79) suited to criteria in samples taken at morning just after disinfection process but hospital 3 (134.8 ± 15.02) did not in nursery section. The quality of air sampled at evening in hospital 1 (78.89 ± 5.675), hospital 2 (80.67 ± 6.24) and hospital 3 (228.1 ± 25.86) did not adhere to requirements.[22] Absence of unidirectional flow system in different sections of all three hospitals might be a major factor for such a rise microbial count. Moreover, disinfection process done at morning time was not performed at evening time in three hospitals. Among three hospitals, hospital 3 had much increased number of viable microorganisms in OPD, ICU and nursery sections as compared to hospital 1 and hospital 2. As the hospital 3 was a teaching hospital, more number of individuals visited it. Movements associated with high number of visitors increased the atmospheric microbial density.

Chemical tests performed to identify microorganisms in this study showed the presence of *E. coli, S. aureus, P. aeruginosa* and *K. pneumonia*. *E. coli* is an emerging nosocomial pathogen in health care system and can cause urinary tract infection, pneumonia, septicemia and gastroenteritis.[23,24] *S. aureus* is the most notorious pathogen for nosocomial infections and can cause diseases in immunocompromised hospitalized patients.[25] A study showed that *P. aeruginosa* is responsible

for 11% of all nosocomial infections and may cause surgical and wound infections, pneumonia, cystic fibrosis and bacteremia.[26] *K. pneumonia,* the eighth significant pathogen in healthcare settings, may be involved in neonatal septicemia, pneumonia, wound infections and septicemia.[27] Presence of nosocomial infections associated pathogens in the atmospheric environment of three hospitals imply a great risk for hospitalized patients at Pakistan. Proper control measures become mandatory to reduce air borne microorganism's count. Guidance for implying and maintaining high hygiene environment at hospital levels should be encouraged.

The outcome of present study was that microbe contaminated air might be a source of nosocomial infection in hospital environments at Pakistan. Our studies showed that quality of atmospheres of three hospitals varies depending upon the number of individuals visiting hospitals. Systematic vigilance, disinfection process updating, infection control practices among health care workers and limitation of patients relative might be the best strategies to keep hospitals environment at safe level. Further studies are necessary to reveal the risk factors associated with nosocomial infections at developing countries so that adequate measures can be taken to reduce the mortality among these patients.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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The Synthesis, Antimicrobial Activity Studies, and Molecular Property Predictions of Novel Benzothiazole-2-Thione Derivatives

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ABSTRACT

Benzothiazoles and 2-mercaptobenzothiazoles are important classes of bioactive organic scaffolds possessing antibacterial, antifungal, antitubercular, antiinflammatory, antidiabetic, and antimalarial properties. In recent years, prediction of drug-likeness, molecular, absorption, distribution, metabolism, and excretion (ADME) properties using in silico techniques has become a standard procedure for the evaluation of molecules in terms of their potential clinical use. In this study, compounds structured 6-benzoyl-3-substitutedmethylbenzo[d]thiazole-2(*3H*)-thione were synthesized using the Mannich reaction starting from 2-mercaptobenzothiazole. The antibacterial and antifungal activities of these compounds were determined against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida krusei,* and *Candida parapisilosis* using a broth microdilution method. An additional analysis was undertaken using the in silico technique to predict the drug-likeness, molecular, and ADME properties of these molecules. Among all the compounds, respectively, Compounds 1-4 and 6-11 exhibited good minimum inhibition concentration values against *Staphylococcus aureus* and Candida species with promising predicted properties.

Keywords: Benzothiazole-2-thione, ADME, antifungal, *Candida albicans*, molecular properties, in silico

INTRODUCTION

Today, the misuse of antibiotics has become an important global concern in terms of causing antimicrobial resistance. Fluoroquinolone resistance of *Escherichia coli* is very widespread and this treatment is now ineffective in more

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than half of the patients. Furthermore, it is estimated that people with methicillin-resistant *Staphylococcus aureus* (MRSA) are 64% more likely to die of this infection compared to people with a non-resistant form. The situation is similar for resistance in *Klebsiella pneumoniae*, Enterobacteriaceae and HIV infections, gonorrhea, tuberculosis, malaria, and influenza. Clearly, development of new active compounds for the antimicrobial resistance of bacteria, fungi, viruses, and parasites is a priority[1].

Benzothiazole (BTA) analogs are one of the most versatile classes of compounds which are a common and integral feature of a variety of natural products and pharmaceutical agents. BTA derivatives have attracted continuing interest due to their diverse biological activities including anticancer, antimicrobial, anticonvulsant, antiviral, antitubercular, antimalarial, antihelminthic, analgesic, antiinflammatory, antidiabetic, and fungicidal activities[2].

Previous studies have shown that compounds derived from the 2nd, 5th, and 6th positions of benzothiazole and 2-mercaptobenzothiazole structures by various functional groups are effective antimicrobial and antifungal agents. In addition, substitution of electron withdrawing groups such as amino, nitro, trifluoromethyl, and halogens especially at the 6th position enhances the antimicrobial and antifungal activities[3-13]. In their literature review, Keri et.al.[2] and Azam and Suresh[14] found that the pharmacological activity of these systems has been widely investigated and found efficient.

In a very early study, Halasa and Smith suggested that the benzothiazole-2-thiol ring system was in a tautomeric form with benzothiazole-2-thione (Figure 1) and Michael/Mannich reactions could easily be performed to produce N-substituted benzothiazole-2-thione compounds with excellent yields[15].

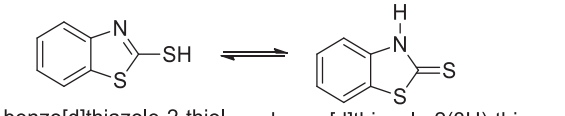


Figure 1. Tautomeric forms of the benzothiazole-2-thiol ring system

Mannich bases have been reported to exhibit antifungal and antimicrobial activities when connected to various ring systems[16]. Furthermore utilizing the antibacterial[17-20], antispasmodic[21], and antitubercular activities[22] of the benzothiazole-2-thione ring system, Varma et al. synthesized a series of N-substituted-5-(hydrogen/chloro)benzothiazole-2-thiones and found that the compounds were active over *Escherichia coli* and *Staphylococcus aureus*[23-25].

Electronic behavior of benzothiazole-2-thione is similar to that of the benzothia-

zol-2-one ring. Studies on the latter have shown that acylation of this ring system at the 6th position can be achieved either by Friedel-Craft conditions or through the reaction of carboxylic and polyphosphoric acids[26]. Considering these findings, addition of a moderately electron withdrawing group such as benzoyl to the 6th position of the benzothiazole-2-thione ring system and performing a Mannich reaction with the ring nitrogen can result in products with antimicrobial or antifungal activities.

In this study, we synthesized 6-benzoyl-3-substitutedmethylbenzo[d]thiazole-2(*3H*)-thione derivatives. We also determined the antibacterial and antifungal activities of the compounds against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida krusei*, and *Candida parapisilosis* using a broth microdilution method. Furthermore, their absorption, distribution, metabolism, and excretion (ADME), drug-likeness, and molecular properties were predicted by in silico techniques.

METHODOLOGY

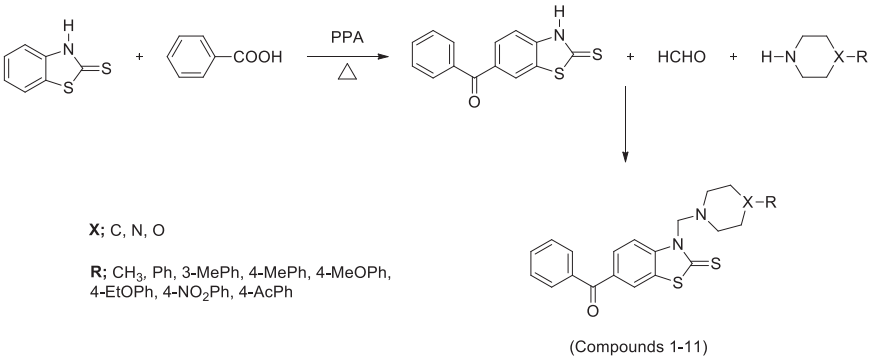
Chemistry

All the chemicals were purchased from Aldrich Chemical Co. (Steinheim, Germany). Melting points were determined with a Mettler-Toledo FP62 capillary melting point apparatus (Columbus, OH, USA). IR spectra (KBr) were recorded on a PerkinElmer Spectrum One FT-IR spectrometer (Waltham, MA, USA) and 1H-NMR spectra were obtained by Bruker DPX-400, 400 MHz High Performance Digital FT-NMR. All the chemical shift values were recorded as δ (ppm). Mass spectra were recorded using an Agilent 1100 series LC/APCI/MS 1946 G spectrometer in the negative ionization mode. The purity of the compounds was checked by thin-layer chromatography on silica gel-coated aluminum sheets (Merck, 1.005554, silica gel HF254–361, Type 60, 0.25 mm; Darmstadt, Germany). Elemental analyses were performed with a Leco CHNS 932 analyzer (Leco Corp., MI, USA) and found to be within ± 0.4 % of the theoretical values for C, H, and N.

General synthesis

A solution of 2-mercaptobenzothiazole (40 mmol) in 150 mL of polyphosphoric acid was reacted portion wise with benzoic acid (50 mmol) under mechanical stirring and further heated to 130 °C for 12 hr. After cooling, the reaction mixture was poured onto ice-water. The precipitated 6-benzoylbenzo[d]thiazole-2(*3H*)-thione was filtered, washed with ice-cold water, and recrystallized from ethanol. Then, 6-benzoylbenzo[d]thiazole-2(*3H*)-thione (0.5 mol) was suspended in 20 ml of ethanol followed by the addition of first 7.5 ml of 37 % formalin to this

suspension and then an appropriate secondary amine (0.05 mol). The reaction mixture was stirred at room temperature for 4 hr with occasional warming on a water bath. After cooling and filtering, the final products were collected from the reaction vessel, washed with cold ether, and recrystallized from the ethanol-acetone mixture as solids (Scheme 1).



Scheme 1. Synthetic pathway followed for the preparation of 3-substitutedmethyl-6-benzoylbenzo[d] thiazole-2(*3H*)-thione derivatives (Compounds 1-11)

6-Benzoyl-3-(piperidin-1-ylmethyl)benzo[d]thiazole-2(3*H*)-thione (Compound 1)

Yield 60%, M.p.: 213 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3000 (CH, aromatic), 2795 (CH, aliphatic), 1680 (C=O). ^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 1.5- 1.6 (m, 6H, Pip H), 2.45 (t, *J=7.10* Hz, 4H, Pip H), 4.15 (s, 2H, -CH_2-), 7.43–7.59 (m, 3H, Ar H), 7.72 (d, *J=8.2* Hz 2H, Ar H), 7.80 (d, *J=9.5* Hz, 2H, Ar H), 8.50 (s, 1H, ArH) ppm. MS 368.1 (M^+). Anal. calcd for $C_{20}H_{20}N_2OS_2$: C, 65.18; H, 5.47; N, 7.60. Found: C, 65.16; H, 5.44; N, 7.62.

6-Benzoyl-3-(morpholin-4-ylmethyl)benzo[d]thiazole-2(*3H*)-thione (Compound 2)

Yield 50%, M.p.: 186 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3005 (CH, aromatic), 2805 (CH, aliphatic), 1685 (C=O). ^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.50 (t, *J=7.00* Hz, 4H, Mor), 3.65 (t, *J=7.00* Hz, 4H, Mor), 4.14 (s, 2H, -CH_2-), 7.45–7.61 (m, 3H, Ar H), 7.74 (d, *J=8.2* Hz, 2H, Ar H), 7.82 (d, *J=9.5* Hz, 2H, Ar H), 8.52 (s, 1H, ArH) ppm. MS 370.0 (M^+). Anal. calcd for $C_{19}H_{18}N_2O_2S_2$: C, 61.60; H, 4.90; N, 7.56. Found: C, 61.58; H, 4.88; N, 7.55.

6-Benzoyl-3-(piperazin-1-ylmethyl)benzo[d]thiazole-2(*3H*)-thione (Compound 3)

Yield 70%, M.p.: 208 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3305 (NH, Pip.), 3008 (CH, aromatic), 2810 (CH, aliphatic), 1689 (C=O).^{1}H-NMR (400 MHz, DMSO-

d6, δ): 1.98 (s, 1H, Ppz NH), 2.37 (t, 4H, Ppz H), 3.42 (t, 4H, Ppz H), 4.15 (s, 2H, -CH_2-), 7.43–7.59 (m, 3H, Ar H), 7.72 (d, *J=8.2* Hz, 2H, Ar H), 7.80 (d, *J=9.5* Hz, 2H, Ar H), 8.50 (s, 1H, Ar H) ppm. MS 369.1 (M^+). Anal. calcd for $C_{19}H_{19}N_3OS_2$: C, 61.76; H, 5.18; N, 11.37. Found: C, 61.73; H, 5.19; N, 11.40.

6-Benzoyl-3-((4-methylpiperazin-1-yl)methyl)benzo[d]thiazole-2(*3H*)-thione (Compound 4)

Yield 78%, M.p.: 220 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3008 (CH, aromatic), 2820 (CH, aliphatic), 1650 (C=O). ^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.26 (s, 3H, Ppz-CH_3), 2.35 (s, 8H, Ppz H), 4.18 (s, 2H, -CH_2-), 7.43–7.59 (m, 3H, Ar H), 7.70 (d, *J=8.2* Hz, 2H, Ar H), 7.79 (d, *J=9.5* Hz, 2H, Ar H), 8.54 (s, 1H, ArH) ppm. MS 383.1 (M^+). Anal. calcd for $C_{20}H_{21}N_3OS_2$: C, 62.63; H, 5.52; N, 10.96. Found: C, 62.65; H, 5.56; N, 10.93.

6-Benzoyl-3-((4-phenylpiperazin-1-yl)methyl)benzo[d]thiazole-2(*3H*)-thione (Compound 5)

Yield 80%, M.p.: 242 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm-1): 3050 (CH, aromatic), 2930 (CH, aliphatic), 1655 (C=O).^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.49 (t, 4H, Ppz H), 2.65 (t, 4H, Ppz H), 4.15 (s, 2H, -CH_2-), 6.78-7.1 (m, 5H, Ppz-Phe), 7.43–7.59 (m, 3H, ArH), 7.72 (d, *J=8.2* Hz, 2H, ArH), 7.80 (d, *J=9.5* Hz, 2H, ArH), 8.50 (s, 1H, ArH) ppm. MS 445.1 (M^+). Anal. calcd for $C_{25}H_{23}N_3OS_2$: C, 67.39; H, 5.20; N, 9.43. Found: C, 67.41; H, 5.23; N, 9.39.

6-Benzoyl-3-((4-(3-methylphenyl)piperazin-1-yl)methyl)benzo[d] thiazole-2(*3H*)-thione (Compound 6)

Yield 70%, M.p.: 190 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3050 (CH, aromatic), 2940 (CH, aliphatic), 1649 (C=O).^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.35 (s, 3H, Phe-CH_3), 2.48 (t, 4H, Ppz H), 3.44 (t, 4H, Ppz H), 4.15 (s, 2H, -CH_2-), 6.60-7.1 (m, 4H, Ppz-Phe), 7.43–7.59 (m, 3H, ArH), 7.72 (d, *J=8.2* Hz, 2H, ArH), 7.80 (d, *J=9.5* Hz, 2H, ArH), 8.50 (s, 1H, ArH) ppm. MS 459.1 (M^+). Anal. calcd for $C_{26}H_{25}N_3OS_2$: C, 67.94; H, 5.48; N, 9.14. Found: C, 67.98; H, 5.44; N, 9.10.

6-Benzoyl-3-((4-(4-methylphenyl)piperazin-1-yl)methyl)benzo[d] thiazole-2(*3H*)-thione (Compound 7)

Yield 74%, M.p.: 215 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3049 (CH, aromatic), 2930 (CH, aliphatic), 1648 (C=O). ^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.34 (s, 3H, Phe-CH_3), 2.48 (t, 4H, Ppz H), 3.44 (t, 4H, Ppz H), 4.15 (s, 2H, -CH_2-), 6.64 (d, *J=9.0* Hz , 2H, Ppz-Phe), 7.05 (d, *J=9.0* Hz, 2H, Ppz-Phe), 7.43–7.59 (m, 3H, ArH), 7.72 (d, *J=8.2* Hz, 2H, ArH), 7.80 (d, *J=9.5* Hz, 2H, ArH), 8.50 (s, 1H, ArH) ppm. MS 459.1 (M^+). Anal. calcd for $C_{26}H_{25}N_3OS_2$: C, 67.94; H, 5.48; N, 9.14. Found: C, 67.90; H, 5.45; N, 9.12.

6-Benzoyl-3-((4-(4-methoxyphenyl)piperazin-1-yl)methyl)benzo[d] thiazole-2(*3H*)-thione (Compound 8)

Yield 70%, M.p.: 252 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3055 (CH, aromatic), 2924 (CH, aliphatic), 1649 (C=O). ^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.48 (t, 4H, Ppz H), 3.44 (t, 4H, Ppz H), 3.84 (s, 3H, -OCH_3), 4.13 (s, 2H, -CH_2-), 6.65-6.81 (m, 4H, Ppz-Phe), 7.43–7.59 (m, 3H, ArH), 7.72 (d, *J=8.2* Hz, 2H, ArH), 7.80 (d, *J=9.5* Hz, 2H, ArH), 8.50 (s, 1H, ArH), ppm. MS 475.1 (M^+). Anal. calcd for $C_{26}H_{25}N_3O_2S_2$: C, 65.66; H, 5.30; N, 8.83. Found: C, 65.63; H, 5.33; N, 8.80.

6-Benzoyl-3-((4-(4-ethoxyphenyl)piperazin-1-yl)methyl)benzo[d] thiazole-2(*3H*)-thione (Compound 9)

Yield 85%, M.p.: 194 °C, white solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3040 (CH, aromatic), 2920 (CH, aliphatic), 1652 (C=O). ^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 1.32 (t, *J=8.0* Hz, 3H, -CH_3), 2.48 (t, 4H, Ppz H), 3.44 (t, 4H, Ppz H), 4.09 (q, 2H, -OCH_3), 4.13 (s, 2H, -CH_2-), 6.65-6.81 (m, 4H, Ppz-Phe), 7.43–7.59 (m, 3H, ArH), 7.72 (d, *J=8.2* Hz, 2H, ArH), 7.80 (d, *J=9.5* Hz, 2H, ArH), 8.50 (s, 1H, ArH), ppm. MS 489.2 (M^+). Anal. calcd for $C_{27}H_{27}N_3O_2S_2$: C, 66.23; H, 5.56; N, 8.58. Found: C, 66.27; H, 5.60; N, 8.61.

6-Benzoyl-3-((4-(4-nitrophenyl)piperazin-1-yl)methyl)benzo[d]thiazole-2(*3H*)-thione (Compound 10)

Yield 55%, M.p.: 234 °C, white-reddish solid. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3020 (CH, aromatic), 2918 (CH, aliphatic), 1649 (C=O). ^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.49 (t, 4H, Ppz H), 3.45 (t, 4H, Ppz H), 4.13 (s, 2H, -CH_2-), 7.02 (d, 2H, Ppz-Phe), 7.43–7.59 (m, 3H, ArH), 7.72 (d, *J=8.2* Hz, 2H, ArH), 7.80 (d, *J=9.5* Hz, 2H, ArH), 8.50 (s, 1H, ArH), 8.70 (d, 2H, Ppz-Phe) ppm. MS 490.1 (M^+). Anal. calcd for $C_{25}H_{22}N_4O_3S_2$: C, 61.20; H, 4.52; N, 11.42. Found: C, 61.18; H, 4.49; N, 11.39.

6-Benzoyl-3-((4-(4-acetylphenyl)piperazin-1-yl)methyl)benzo[d]thiazole-2(*3H*)-thione (Compound 11)

Yield 64%, M.p.: 263 °C, white. IR (KBr) $\bar{\upsilon}_{max}$ (cm^{-1}): 3024 (CH, aromatic), 2926 (CH, aliphatic), 1655 (C=O). ^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.48 (t, 4H, Ppz H), 2.50 (s, 3H, CH_3), 3.44 (t, 4H, Ppz H), 4.13 (s, 2H, -CH_2-), 6.85 (d, 2H, Ppz-Phe), 7.43–7.59 (m, 3H, ArH), 7.72 (d, *J=8.2* Hz, 2H, ArH), 7.77 (d, 2H, Ppz-Phe), 7.80 (d, *J=9.5* Hz, 2H, ArH), 8.50 (s, 1H, ArH), ppm. MS 487.1 (M^+). Anal. calcd for $C_{27}H_{25}N_3O_2S_2$: C, 66.50; H, 5.17; N, 8.62. Found: C, 66.53; H, 5.15; N, 8.61.

Microbiological Screening

The following test microorganisms were obtained from LGC Standards GmbH (Wesel, Germany): *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 60193, *Candida krusei* ATCC 28870, and *Candida parapisilosis* ATCC 90018. All the synthesized compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution at 10 mg/mL.

Broth microdilution method

The minimal inhibition concentration (MIC) values (μg/mL) for the organisms were determined using the methods recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines[27-28]. The antimicrobial effects of the substances against all the microorganisms were quantitatively tested in broth media using double dilution. The antibacterial and antifungal assays were performed in a Mueller Hinton broth (Difco) at pH 7.3 and a buffered yeast nitrogen base (Difco) at pH 7.0, respectively. MIC was defined as the lowest concentration with no bacterial or fungal growth. Carbenicillin (10 μg/mL) and fluconazole (10 μg/mL) were prepared as stocks, then diluted in a range from 10 to 0.5 μg/mL using DMSO, and tested as standard antibacterial and antifungal drugs, respectively. For all the compounds, the tested dilutions ranged from 128 to 0.5 μg/mL using DMSO as the solvent. The control samples prepared with the amounts of DMSO used in the dilutions did not show any inhibitory activity under these conditions.

Table 1: Antimicrobial activity of compounds using microdilution (MIC, μg/mL)

Compound	*Staphylococcus aureus*	*Escherichia coli*	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Candida albicans*	*Candida krusei*	*Candida parapisilosis*
1	8	128	128	256	32	64	64
2	8	256	128	256	32	64	64
3	16	128	128	128	32	64	64
4	4	64	256	128	64	128	64
5	64	64	256	256	64	32	32
6	64	128	256	256	64	32	16
7	128	128	256	128	64	16	8
8	128	64	128	128	16	8	4
9	64	64	128	128	16	8	4
10	32	64	256	256	8	16	16
11	64	64	128	256	8	8	4
Carbenicillin	4	8	32	32			
Fluconazole					0.5	64	4

Prediction of drug-likeness, molecular and ADME properties

All the molecules were prepared in 3D using the LigPrep module of Maestro (Schrodinger Inc.). The ADME properties (46 molecular descriptors) were determined using the QikProp program (Schrödinger 2015-3) in the normal mode. QikProp generates physically relevant descriptors, which are then used to perform ADME predictions. An overall ADME-compliance score, the drug-likeness parameter (indicated by #stars), was used to assess the pharmacokinetic profiles of the compounds. The #stars parameter (ranging from 0 to 5) indicates the number of property descriptors computed by QikProp that fall outside the optimum range of values for 95% of known drugs. The following descriptors were predicted: Central nervous system (CNS) activity (from –2 for inactive to +2 for active); octanol/water partition coefficient, logPo/w (–2.0 to 6.5); IC_{50} value for the block of HERG K^+ channels, log *HERG* (concern < –5); Caco-2 cell membrane permeability in nm s^{-1}, PCaco (: < 5 low to > 100 high); logarithm of the predicted blood/brain barrier partition coefficient, log B/B (-3.0 to 1.0); apparent Madin-Darby canine kidney cell permeability (PMDCK) that mimic the blood-brain barrier for non-active transport in nm s^{-1}, PMDCK (< 25 poor to > 500 great); skin permeability, log K_p (–8.0 to –1.0); logarithm of binding constant to human serum albumin, log K_{HSA} (-1.5 to 1.2); qualitative human oral absorption (HOA) (1: low, 2: medium, 3: high); percent of HOA (>80%: high, <25%: poor) (Table 2).

Table 2: The calculated drug-likeness, molecular properties and ADME predictions for Compounds 1-11 using QikProp

Compound	#stars	CNS	logPo/w	logHERG	PCaco	logBB	PMDCK	logKp	$logK_{HSA}$	HOA	%HOA
1	0	2	3.729	-6.521	781.726	0.463	1698.396	-3.241	0.187	3	100
2	0	2	2.617	-6.047	820.341	0.513	1731.346	-3.207	-0.395	3	94.425
3	0	2	2.325	-7.064	105.042	0.59	205.004	-5.823	0.042	3	76.735
4	0	2	2.388	-7.238	175.945	0.793	329.151	-5.362	-0.248	3	81.116
5	0	1	4.822	-7.772	804.78	0.437	1673.878	-2.564	0.528	3	100
6	0	1	4.933	-7.443	756.618	0.412	1611.668	-2.871	0.621	3	100
7	0	1	5.244	-7.751	824.606	0.433	1750.35	-2.729	0.738	3	96.89
8	0	1	4.75	-7.67	725.135	0.297	1376.039	-2.734	0.469	3	100
9	0	1	5.204	-7.908	723.683	0.211	1373.061	-2.651	0.63	3	95.636
10	1	0	4.082	-7.722	82.714	-0.794	143.123	-4.598	0.458	3	85.166
11	0	1	4.023	-7.692	231.066	-0.288	399.738	-3.742	0.249	3	92.808

RESULTS

Eleven 6-benzoyl-3-substitutedmethylbenzo[d]thiazole-2(*3H*)-thione derivatives were successfully synthesized by the Mannich method giving yields between 50 and 85%. In the IR spectra, all the compounds had a strong C = O stretching band for the benzoyl group at 1648–1689 cm^{-1}. The ^{1}H-NMR spectra of the compounds showed that the protons belonging to the 6-benzoylbenzo[d] thiazole-2(*3H*)-thione ring system exhibited similar properties to those reported by previous studies[15, 29]. All the other protons were observed according to the expected chemical shift and integral values. The molecular ion peaks (M^{+}) of the compounds were examined under electron ionization and confirmed the molecular weights of the compounds. The MIC values of Compounds 1-11 were evaluated for their antibacterial and antifungal activities using carbenicillin and fluconazole as a standard for the microorganisms. For practical purposes, the MIC values ≤ 16 μg/mL were considered to be active for the evaluation of the results. None of the synthesized compounds was found to be active against *Escherichia coli, Escherichia. faecalis,* and *Pseudomonas. aeruginosa* as much as the standard carbenicillin. According to the results of *Staphylococcus aureus*, Compounds 1-4 showed an antibacterial activity comparable to the same standard. Similarly, for *Candida albicans,* Compounds 8-11 showed some activity but performed worse than fluconazole. Compounds 7-11 had a strong activity against *Candida krusei* resulting in similar inhibition concentrations of 8 to 16 μg/mL, which was better than fluconazole with the inhibition concentration of 64 μg/mL. In the broth dilution experiments, Compounds 6, 7, and 10 were found to have an MIC of 8 to 16 μg/mL, which had a moderate activity against *Candida parapisilosis*. Another significant result was that Compounds 8, 9, and 11 had MIC values of 4 μg/mL presenting the same anti-fungal activity as fluconazole. Interestingly, compounds with either small substituents at the 4th position or non-substituted 6-Benzoyl-3-(piperidinyl/piperazinyl/morpholin-4-ylmethyl) benzo[d] thiazole-2(*3H*)-thione derivatives were active against *Staphylococcus aureus***.** On the contrary the aromatic functional groups containing complex substituents were more active against Candida species.

In this study, the drug-likeness, molecular and ADME properties of all compounds were promising presenting a drug-like/lead-like profile according to their #stars rankings. The #stars rankings were 0 for all the compounds except Compound 10 (1). The combinations of HOA values being mostly around 3, %HOA values ranging from 76.73 to 100%, all PCaco values being high and log Kp values varying between -2.54 and -5.82 indicate that these compounds can be effectively used in both oral and topical preparations. The $\log K_{HSA}$ values varying between -0.35 and +0.45 indicates that the proposed molecules can freely

circulate and easily traverse cell membranes without binding to human serum albumin. For determining the cardiac toxicity of drugs in the early stages of drug discovery, HERG K^+ channel blockage activity is very important [30]. The logHERG values of the compounds predicted with the in silico method were between -6.04 and -7.90; thus, they can be considered safe for human use. The blood/brain partition coefficient (logBB), PMDCK, and logPo/w values are useful to determine the penetration capacity of a compound from blood–brain barrier. The values predicted for these parameters of the synthesized compounds were within the ranges defined for 95% of drugs. Moreover, the predicted CNS value of the compounds was between 0 and 2 indicating medium to high activity.

The results of the activity analyses showed that the synthesized 6-benzoyl-3-substitutedmethylbenzo[d]thiazole-2(*3H*)-thione derivatives could be considered as potential effective antifungal agents against Candida species. Further studies are necessary to confirm and validate the predictive results based on actual experiments.

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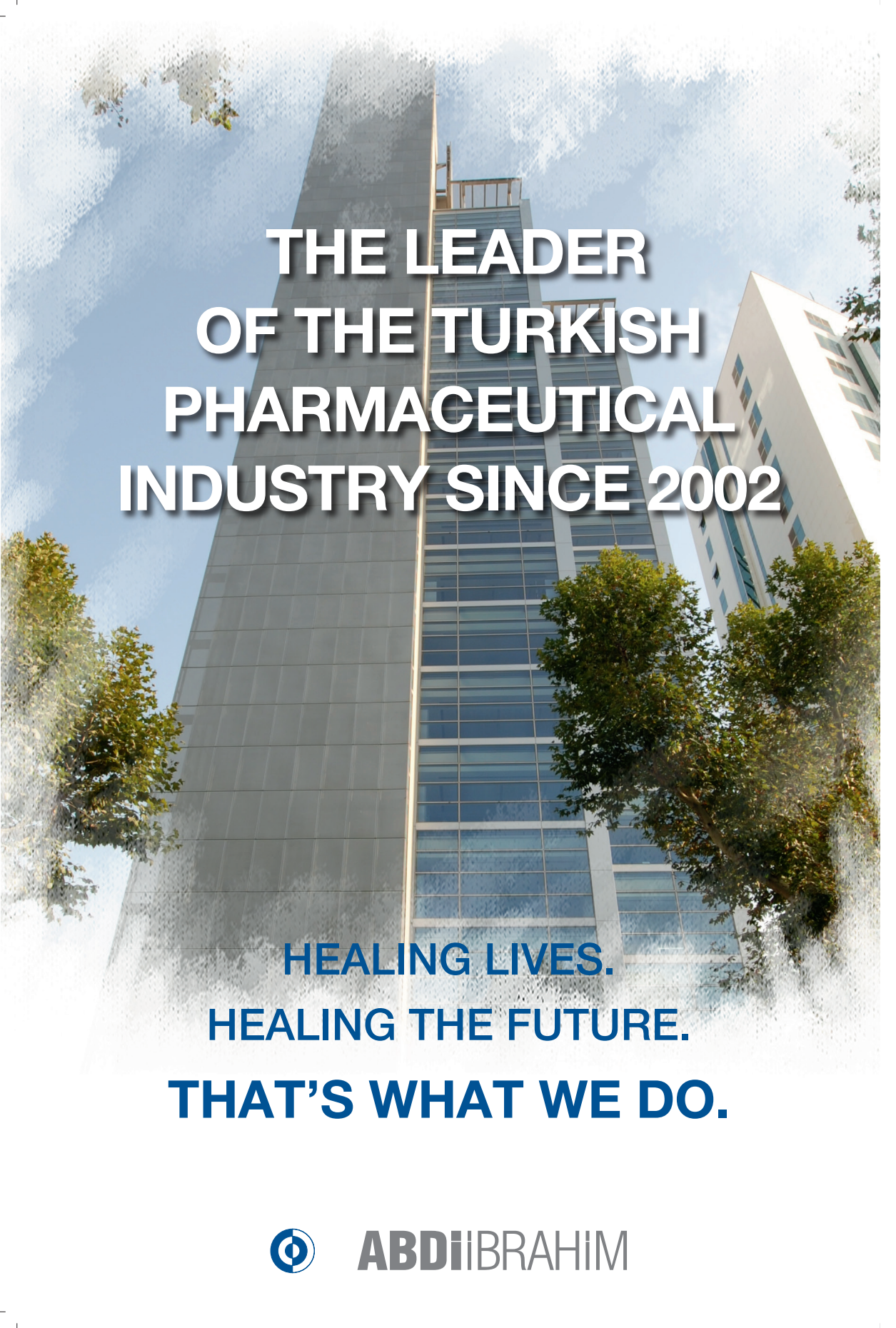
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The Effects of Some Imidazopyrazine Derivatives on Telomerase Inhibition, mtDNA Damage and mtDNA Copy Number

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ABSTRACT

Imidazopyrazine derivatives have been studied for their curative effects on some diseases like cancer and neurological problems; also, some of these molecules have been patented. Primary human cells exhibit limited replicative potential but the cancer cells divided unlimitedly with passage in culture. This immortality is mainly a result of telomerase activity. We investigated the possible telomerase inhibitor effect and possible mtDNA damage action of five imidazopyrazine derivatives. Telomerase activities were measured by the PCR-ELISA based TRAP method and mtDNA damage assays were achieved by quantitative PCR. We used zebrafish as a model organism for our research. In the application of 6-(4-Metylphenyl-8-(4-chlorophenyl)imidazo[1,2-a]pyrazine ($C_{19}H_{14}N_3Cl$), it was determined that this compound inhibit telomerase activities to a statistically significant degree. The obtained results from molecular docking studies also supported the experimental results. Accordingly, this compound has the probability to be used as an anti-cancer agent after detailed studies.

Keywords: Imidazopyrazine derivatives, Telomerase inhibition, mtDNA damage, mtDNA copy number, anticancer drugs

INTRODUCTION

The use of chemotherapy to treat cancer began at the start of the 20th century. In the 1960s and early 1970s, chemotherapy could cure acute childhood leukemia Hodgkin's disease. These advances of the drug development created hope for to cure cancers. Today, targeted therapies provide a broad perspective for treatment of more diseases. [1]

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The molecules that were synthesized by researchers in the past have become important drugs today. Imidazopyrazine derivatives have been studied for their curative effect on some diseases like cancer and neurological problems; also, some of these molecules have been patented (US7189723 B2[2], WO2002010170 A1[3], US7259164 B2[4], US7393848 B2[5]).

We investigated the possible telomerase inhibitor effect of five imidazopyrazine derivatives synthesized by Demirayak and Kayagil. According to their research, these molecules have cytotoxic effects on some types of cancer cells.[6] There were no data about the mechanism of cytotoxic action but these imidazopyrazine derivatives have structural similarities with some telomerase inhibitors.

Telomeres are specialized functional complexes that protect the ends of eukaryotic chromosomes. The telomeric DNA sequences are tandem repeats of a short hexameric sequence unit in most species.[7] Greider and Blackburn identified a specialized DNA polymerase in extracts from the *Tetrahymena*, that extends the chromosome ends in eukaryotes.[8] Telomerase adds multiple copies of certain DNA unit to the terminal portion of one strand of the repeat tract.[7]

Telomerase is a very important enzyme for the aging process and carcinogenesis. Primary human cells exhibit limited replicative potential but the cancer lines divided indefinitely with passage in culture.[9] Human cancer cells must compensate the progressive loss of telomeric DNA by cell division to grow indefinitely.[10] This immortality is mainly a result of telomerase activity. Telomerase is expressed in more than 85% of cancer cells[11], but in some cells, the telomere length could be maintained in the absence of telomerase. It has been revealed that one or more alternative telomerase-independent mechanisms exist in human cells.[10]

Telomere shortening may cause aging and death. Some evidence suggests that the progressive loss of telomeric repeats of chromosomes may function as a molecular clock that triggers senescence.[12-14] Epidemiological researches show that shorter telomeres may be associated with many age related diseases.[12] Studies showed that the telomerase gene therapy in mice delays aging and increases longevity.[15,16]

The aim of this study is the investigation of the telomerase inhibitor effects of some diarylimidazopyrazine derivatives and to search for possible mtDNA damage action.

Toxic materials may generate damage on mitochondrial DNA (mtDNA).[17-19] mtDNA damage may trigger mitochondrial dysfunction.[20] Entire mitochondrial genome codes for genes are expressed while nDNA contains a large amount of non-transcribed sequences. Because of that the damage to mtDNA could be po-

tentially more important than the nDNA damage. Also mtDNA is continuously replicated even in terminally differentiated cells. Therefore, somatic mtDNA damage potentially causes more adverse effects on cellular functions.[21]

We used zebrafish as a model organism for our research. The zebrafish (*Danio rerio*) has been developed as a powerful model for aging, cancer, developmental biology and some aging-related diseases.[22] One of the best model organisms for telomerase studies is the zebrafish. Conversely to the inbred laboratory mouse, zebrafish have heterogeneous telomeres of human-like length and zebrafish telomeres shorten with age.[23]

METHODOLOGY

Maintenance of Zebrafish

We used the spleen of zebrafish for measuring telomerase enzyme activity and liver for mtDNA damage. Zebrafish (*Danio rerio*) were maintained at 24±2 °C with a light/dark cycle of 14:10 hours and they were fed with dry flake food. The fish were anesthetized with ice before injections and excising of organs (10 fishes for each group). All zebrafish applications were approved by the Ethical Committe of the Mehmet Akif Ersoy University (27.01.2014/57 and 24.02.2015/114).

Diarylimidazopyrazine Derivatives

We investigated the possible telomerase inhibitor effect of five imidazopyrazine derivatives originally synthesized by Demirayak and Kayagil (2005). Diarylimidazopyrazine derivatives that used in this study were coded as 2j, 2m, 2n, 2o and 2p (Table 1 and Figure 1). Diarylimidazopyrazine derivatives were dissolved in DMSO. We decided 10^{-5} M concentration for these molecules according to results of cytotoxic tests achieved by NCI (National Cancer Instıtute of USA)[6]. Ten fishes were analyzed from each group. Compounds and DMSO were injected as 7 µl to the fishes. After 150 minutes, spleens and livers were excised.

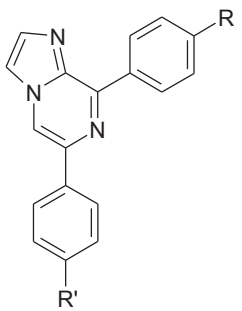

Figure 1. Imidazopyrazine derivatives

Table 1. Diarylimidazopyrazine derivatives that used in this study.

Compounds	R	R'
2j / 6-(4-Methylphenyl)-8-(4-methoxyphenyl)imidazo[1,2-a]pyrazine	OCH_3	CH_3
2m / 6-phenyl-8-(4-chlorophenyl)imidazo[1,2-a]pyrazine	Cl	H
2n / 6-(4-Methylphenyl)-8-(4-chlorophenyl)imidazo[1,2-a]pyrazine	Cl	CH_3
2o / 6-(4-Methoxyphenyl)-8-(4-chlorophenyl)imidazo[1,2-a]pyrazine	Cl	OCH_3
2p / 6-(4-Chlorophenyl)-8-(4-chlorophenyl)imidazo[1,2-a]pyrazine	Cl	Cl

Telomerase Assay

Telomerase activities were measured by Roche TeloTAGGG Telomerase PCR ELISA kit, according to the manufacturer's instructions. This kit allows highly specific amplification of telomerase-mediated elongation products combined with nonradioctive detection following an ELISA protocol. Relative Telomerase Activity (RTA) values were calculated for mg/ml protein. Protein values were determined by the Bradford method (Bradford Reagent SIGMA B 6916).

Determination of mtDNA Damage and Copy Number

SIGMA G1N350 Genomic DNA kits were used for total DNA isolation using the methods indicated in the technical bulletin. Invitrogen (Molecular Probes) Pico Green dsDNA quantitation dye and QUBIT 2.0 fluorometer were used for template DNA quantitation and for the fluorometric analysis of PCR products. A crucial step in the QPCR method is the adjusting the concentration of DNA sample. The accuracy of the assay relies on initial template quantity because all of the samples must have the same amount of DNA. The Pico Green dye has not only proven to be an efficient method for template quantitation but also for PCR product analysis.[24,25] DMSO (in a volume equivalent to 4% of total volume) was added to 5 ng of template total DNA in each PCR tube. Thermostabil polymerase used was Thermo Phire hot start II DNA polymerase.

Primers were designed for zebrafish *(Danio rerio)* mtDNA small fragment (95 bp) as:

5' CCCATACTAAAAGCACGCCC 3'

5' CCAGCAACCCTTATTTCGGG 3'

Primers were designed for zebrafish *(Danio rerio)* mtDNA large fragment (10403 bp) as:

5' ACCCATGCCCAAGAGATCAA 3'

5' TTTTCGAGTCACCGGTCTCA 3'

For long fragment PCR amplification, DNA was denatured initially at 98°C for one minute; the material then underwent 21 PCR cycles of 98°C for 10 seconds, 60°C for 45 seconds, and 69°C for five minutes. Final extension was allowed to proceed at 69°C for five minutes.

For small fragment PCR amplification, DNA was denatured initially at 98°C for one minute; the material then underwent 21 PCR cycles of 98°C for 10 seconds, 58°C for 45 seconds, and 72°C for 10 seconds. Final extension was allowed to proceed at 72°C for two minutes.

The QPCR method was used to measure mtDNA damage. The lesion present in the DNA blocked the progression of any thermostable polymerase on the template, so a decrease in DNA amplification was observed in damaged templates. The QPCR method is highly sensitive and useful to measurements of DNA damage and repair. mtDNA damage was quantified by comparing the relative efficiency of amplification of long fragments of DNA and normalizing this to gene copy numbers by the amplification of smaller fragments, which have a statistically negligible likelihood of containing damaged bases. [17,24,26,27] To calculate normalized amplification, the long QPCR values were divided by the corresponding short QPCR results to account for potential copy number differences between samples (the mtDNA/total DNA value may be different in the 5-ng template of total DNA in each PCR tube). The copy number results do not indicate damage.

Computational Methods

AUTODOCK 4.0 MGL Tolls package was used for the docking of imidazopyrazine derivatives to the telomerase.[28,29] This program uses a Lamarkian Genetic Algorithm (LGA) for the docking of ligand interactions. Telomerase (PDB Code: 3KYL) and molecule structure within 3D were optimized by UCSF CHIMERA 1.10.2 before docking procedure. Telomerase - molecule interactions is evaluated using grid-based atomic affiniyt potentials. The end of the reaction time is calculated the final free energy of interaction from the dispersion-repulsion energies, directional hydrogen bonding, dispersion screened electrostatic states and desolvation. Atomic solvation parameters and fragmental volumes were assigned to the protein atoms with the source program AutoDock Vina included in the AutoDock 4.0 free program package. In all the docking simulations we used grid maps with 60x60x60 points. A total of 25 runs using LGA were performed in each separate case were the substrates to be docked were free to rotate around their center single bonds.

Statistical Analysis

Minitab Release 13.0 statistical software was used for analysis. The results were estimated with Mann-Whitney Test.

RESULTS AND DISCUSSION

According to the results, 2n code 6-(4-Metylphenyl-8-(4-chlorophenyl) imidazo[1,2-a]pyrazine ($C_{19}H_{14}N_3Cl$) compound inhibited telomerase activities at statistically significant degree (Table 2). Also, 2j, 2m, 2o and 2p compounds slightly decreased telomerase activity, but this inhibition was not statistically significant. The effect of an Imidazo(1,2-a)pyrazine derivative changes according to side groups (R groups) connected to this molecule.[30]

There are many studies which draw attention to anticancer activities of Imidazo(1,2-a)pyrazine derivatives. In their study which was published in 2012, Leng et al. analyzed structure-activity relation with QSAR method and suggested that some of imidazopyrazine derivatives can function as Aurora A kinase inhibitors [31] Mitchell and his team stated that imidazo(1,2-a)pyrazine diaryl urea compounds can function as receptor tyrosine kinase inhibitors. This development raised hopes for them in using for the treatment of various diseases including cancer.[32] When patent studies carried out for these compounds are considered, it can be seen that there are various patents taken as protein kinase inhibitor. In the study of Matthews et al., it is stated that most of the 3,6-di(hetero)diaryl imidazo(1,2-a)pyrazine derivatives are inhibitors of checkpoint kinase 1 (CHK1) and other kinases.[33] Signal ways which regulate mTOR which is another checkpoint kinase are frequently activated in human cancer. There are patents and essays about use of imidazopyrazine derivates as mTOR inhibitor.[34] There are also patent records about use of imidazopyrazine derivatives as spleen tyrosine kinase (SYK) (US20100152159 A1[35], US20120220582 A1[36]). This enyzme is especially regarded important in B cell lymphoma. B cell receptor (BCR) signals activates SYK and other related ways. BCR and molecules activated by them form important targets for cancer treatment.[37] Also there are specific information in some resources that imidasol derivatives inhibit topoisomerase II and induce apoptosis.[38]

In their study which was carried out by Prevost et al. in 2006, it was revealed that BIM-46174 which is an imidazopyrazine derivative functions as an inhibitor of G protein receptor complex in the cell. In this essay it is pointed out G protein receptors which has an important role in triggering and progressing of cancer can be targeted by imidazopyrazine derivatives.[39]

According to results of our study, some of Imidazo[1,2-a]pyrazine derivatives can be used in order to enable telomerase inhibition. In the application of 2n code 6-(4-Metylphenyl-8-(4-chlorophenyl)imidazo[1,2-a]pyrazine ($C_{19}H_{14}N_3Cl$), it was determined that they inhibit telomerase activities at statistically significant degree (Table 2).

Table 2. Relative Telomerase activity, mtDNA relative amplification and mtDNA copy number results.

Groups	Relative Telomerase Activity ± SE	mtDNA Relative Amplification ± SE	mtDNA Copy Number ± SE
Control	0,428 ± 0,167*	2,519 ± 0,204	101,3 ± 8,7
Control 2 (DMSO)	2,265 ± 0,770	1,841 ± 0,377	112,2 ± 10,4
2j (10^{-5} M)	1,408 ± 0,430	2,115 ± 0,455	107,1 ± 5,8
2m (10^{-5} M)	1,825 ± 0,401	1,735 ± 0,392	104,2 ± 7,9
2n (10^{-5} M)	0,551 ± 0,182*	1,568 ± 0,173	85,54 ± 9,2
2o (10^{-5} M)	1,570 ± 0,260	1,602 ± 0,301	97,02 ± 5,6
2p (10^{-5} M)	1,724 ± 0,383	2,050 ± 0,409	109,1 ± 10,1

*Statistically different from control 2 (DMSO) group ($p<0,05$).

The obtained results from computational docking studies also supported the our experimental results. Overall the calculations results are pointed that the compound, 2n, was shown to be more active than the other compounds (Figure 2). Hydrogen bondings and van der waals interactions were made intensely by 2n especially in some regions of active site of telomerase and these H bondings have lower binding energy.

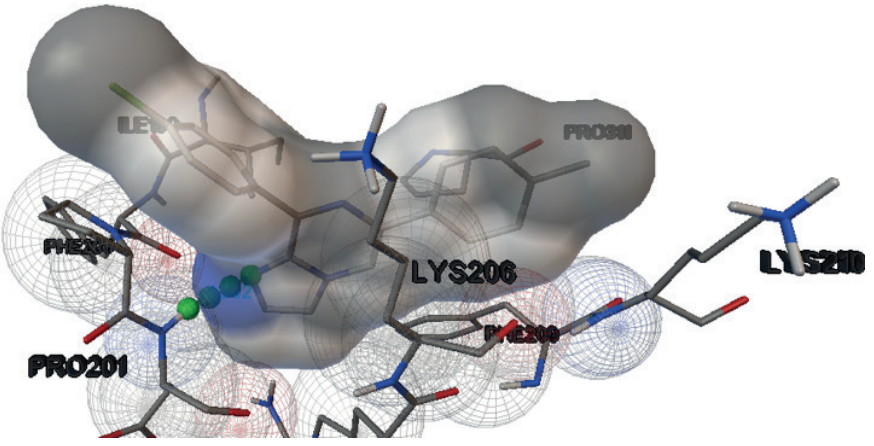

Figure 2. One of the binding positions of 6-(4-Methylphenyl)-8-(4-chlorophenyl) imidazo[1,2-a]pyrazine (2n) with telomerase enzyme (PDB Code: 3KYL).

Clinical studies for telomerase inhibition are currently carried out in three different ways as direct telomerase inhibition through drugs such as Imetelstat (GRN163L), as gene therapy and as immunotherapy practices aiming cells which represent telomerase. In recent studies; some of the telomerase inhibitors were tried on bone marrow, prostate, brain, breast cancer and pancreas cancer cells

and there was decrease in the number of cancer cells.[11] According to our results, it was found out that the compound, 2n, has the probability to be used as an anti-cancer agent after detailed studies.

Moreover, in our study it was observed that dimetylsulphoxide (DMSO) which is used for solving chemical substances, increases telomerase activity. In the study of De Mat et al.; it was determined that cell line which is known to have no/low telomerase activity increase telomerase activity as a result of being exposed to DMSO.[40] In a study which was carried out about differentiation of embrionic stem cell on rats, TERT gene representation raised as a result of dimetylsulphoxide (DMSO) application on each individual and telomerase activity increased.[41]

There were no significant differences among the groups in terms of mtDNA damage or copy number. According to our results, these compounds are not toxic to mtDNA. This is an advantage for the potential usage of the molecules as drugs.

CONCLUSION

The most important disadvantage of chemotherapy drugs used commonly today is that they are not selective. In this way, they have effect on normal healthy cells together with cancer cells. Telomerase inhibition enables more spesific ground. Cancer cells which can be divided limitlessly due to telomerase activation may lose this characteristics through inhibition of telomerase enzyme. According to our results, 2n code 6-(4-Metylphenyl-8-(4-chlorophenyl)imidazo[1,2-a]pyrazine ($C_{19}H_{14}N_3Cl$) compound inhibit telomerase activities at statistically significant degree. It is evaluated that this compound has the probability to be used as an anti-cancer agent after detailed studies. Moreover, the importance of our study increase by making contribution to the literature since cancer disease has no therapy which is effective for all cancer types, therefore it is an important health problem for all people.

ACKNOWLEDGEMENTS

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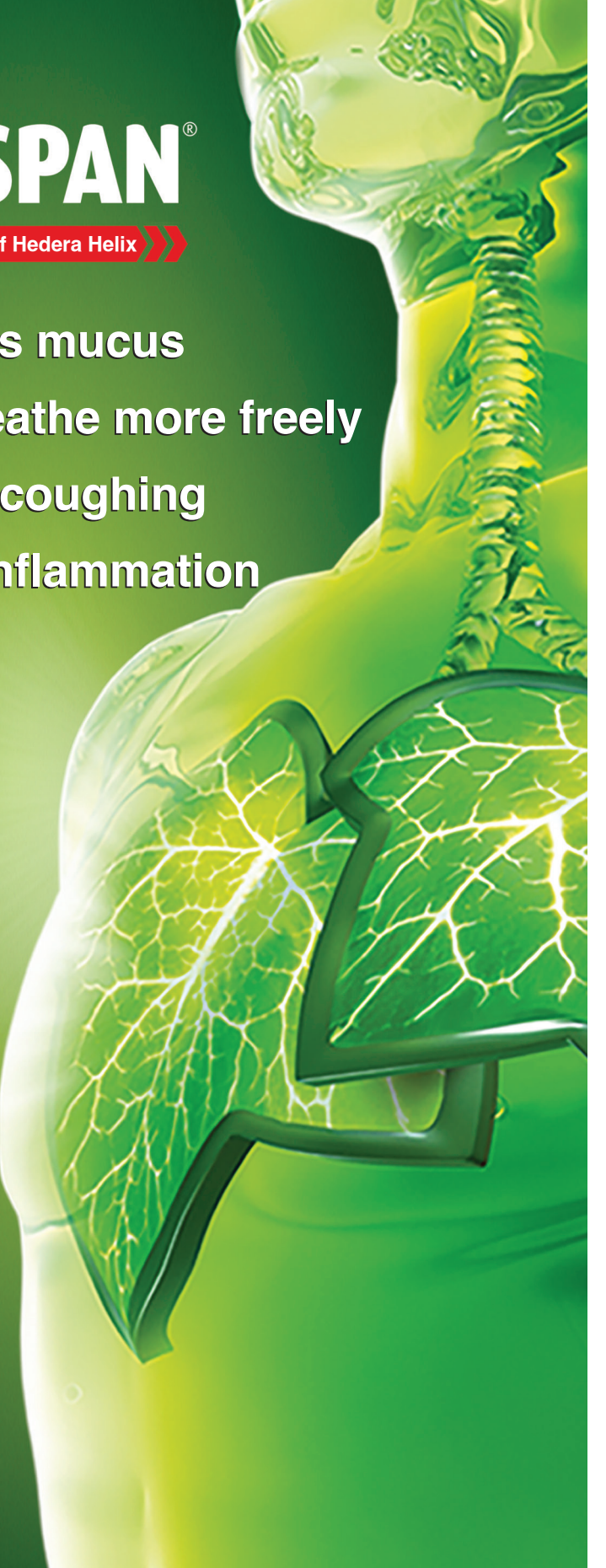

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Classifying Druggability on Potential Binding Sites of Glycogen Synthase Kinase-3β: An In-Silico Assessment

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ABSTRACT

Putative binding sites of glycogen synthase kinase-3β (GSK-3β) have been identified by various computational methods; however, the druggability of these pockets is still unknown. Herein, we assessed a dataset of 24 Protein Data Bank (PDB) crystal structures of GSK-3β using SiteMap to compute the druggability of each identified site. The binding sites were assessed with two site-scoring functions known as the Druggability score (Dscore) and SiteScore (SScore) within SiteMap. An average of eight surface pockets were identified, of which pocket 1 (orthosteric site) and pocket 7 (allosteric site) exhibited ligand-binding characteristics, as analyzed by SiteScore. We further analyzed the druggability of each site with Dscore; pocket 1 proved to be a druggable site, and pocket 7 failed to meet the druggability criteria. The quantitative pocket properties of site 7 were further evaluated to identify plausible reasons for classification as a "difficult" site. In conclusion, these results accurately classified binding sites of GSK-3β.

Keywords: Allosteric sites, Binding sites; Druggability; Glycogen synthase kinase-3; *In silico*

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase that exists in two highly homologous forms: GSK-3α and GSK-3β encoded by two different genes[1-3]. Moreover, in humans, a splice variant (GSK-3β2) has been reported[4]. The role of GSK-3 as a drug target has been implicated in a variety of unmet human diseases, such as Alzheimer's disease (AD)[5–8], bipolar disorder[9,10], various forms of cancers[11,12], diabetes[13], and many other diseases[14–17].

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Over the past years "harder targets" that belong to large super-families like kinases have been addressed in drug discovery. Drug modulators that target the kinase catalytic domain risk serious off-target effects[18]. Moreover, it is still challenging to identify other well defined druggable sites on kinases. Kinetic experiments can depict the mode of inhibition but fail to predict the binding locations on the protein; however, pocket detection algorithms can predict likely binding locations of both orthosteric and allosteric pockets. In this research, the GSK-3β binding sites were assessed with a druggability assessment tool (SiteMap)[19] from Schrödinger, Inc. SiteMap is an energy-based pocket detection algorithm that finds, visualizes, and evaluates protein binding sites. Energy-based methods identify binding sites by docking small organic probes, typically methane or water molecules, on a given target protein to evaluate whether a given protein region interacts favorably[20]. SiteMap is computationally more demanding as this tool provides insights into the physical basis of druggability classification of the target protein sites with high predictive ability.

The X-ray crystal structures reveal three well-known binding sites of GSK-3β: (i) the ATP site, (ii) the substrate binding site, and (iii) the Axin/Fratide binding site. In addition, four allosteric pockets on GSK-3β were recently reported (pockets

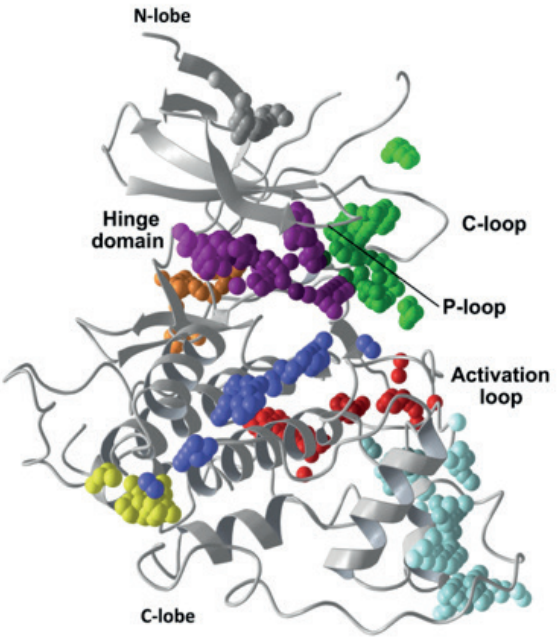

Figure 1. The eight surface pockets of glycogen synthase kinase-3β (GSK-3β) represented as colored spheres identified by SiteMap [Protein Data Bank (PDB) ID: 4NU1; gray cartoon representation]: pocket 1 (pink spheres), pocket 2 (green spheres), pocket 3 (turquoise spheres), pocket 4 (blue spheres), pocket 5 (gray spheres), pocket 6 (brown spheres), pocket 7 (red spheres), and pocket 8 (yellow spheres).

4–7)[21]. Our recent program identified an additional small allosteric pocket at the C-lobe of GSK-3β (pocket 8). Structurally, GSK-3 is a two-domain kinase fold comprising a β-strand domain and an α-helix domain. The residues forming the ATP-binding site (pocket 1) are seated deep between the interface of the α-helix and β-strand domains surrounded by a hinge region and a glycine-rich loop, which is often referred to as "P-loop"[22–25]. The substrate binding site (pocket 2) is surrounded by the C-loop and the activation loop. Both pockets are sandwiched between the interfaces of the N- and C-lobes of GSK-3β. The third known pocket is the Axin/Fratide binding site (pocket 3). Pockets 4–8 are the allosteric sites of the kinase shown in Figure 1.

METHODOLOGY

All computational and molecular modeling studies were carried out using Schrödinger molecular modeling software, version 9.3.5 on a windows Dell workstation.

Protein preparation

Twenty-four different X-ray crystal structures of human GSK-3β were extracted from the Protein Data Bank (PDB) by maintaining selection criteria close to 2.5 angstroms. GSK-3β is crystallized as a homodimer comprising of chains A and B. In a few cases of the crystallographic structures, chain A monomers were crystallized with Axin and Fratide peptides, and in some cases they exist as a homodimer or monomer protein. The dimer structures within each PDB case were separated to retain the chain A monomers as in many cases, site search may generate unphysical sites over the entire dimer structures. Each individual chain A monomers were preprocessed with the Protein Preparation Wizard in the Maestro program, with the following default options selected: "assign bond orders," "add hydrogen atoms," "create zero-order bonds to metals," "create disulphide bonds," and "delete water molecules beyond 5Å from heteroatom groups." The optimal protonation states of each ionizable residue were assigned and the hydrogen-bonding network was optimized for proteins with structural ambiguity. A restrained minimization with an OPLS2005 force field was performed to attain the relaxed state of each refined complex.

Site identification

SiteMap identifies a site as an enclosed region on the protein surface comprising at least 15 site points (default settings)[26]. To locate a site the search uses a grid of points called "site points." Following the default protocol in SiteMap v2.6, a more restrictive definition of hydrophobicity, a standard grid, and the OPLS2005 force field were used.

The refined chain A monomers were then submitted to SiteMap for druggability assessment. To avoid a biased search, all crystallographic water molecules, ligands, metal ions, heteroatom groups, unwanted chains, and peptides were removed prior to the site search. SiteMap was assigned to report up to 10 sites to avoid ambiguity that matches with the known sites of GSK-3β (default settings: 5).

Druggability assessment by SiteMap

The druggability of a protein binding pocket is calculated by various physical descriptors in SiteMap, which were calibrated for submicromolar tight-binding sites[26]. These include; (i) the size of the binding pocket and (ii) the volume of a protein site. The above terms are case sensitive and were not calibrated. The enclosure property (iii) indicates the degree to which a site is sheltered from the solvent (calibrated scores: ~ 0.76). The exposure property (iv) measures the degree of exposure to solvent (calibrated scores ≤ 0.49). The degree of contact (v) measures the relative tightness between the site points and the protein site via van der Walls non-bonding interactions (calibrated scores: ~1.0). The phobic/philic character (vi) is a measure of the relative hydrophobic and hydrophilic nature of the site (calibrated scores: ~1.0). The balance term (vii) indicates the ratio of hydrophobic to hydrophilic character of the site (the calibrated ratio of the two scores is approximately 1.6). The donor/acceptor character (vii) measures the hydrogen-bonding possibility between a ligand and protein site where a ligand donates and the protein accepts hydrogen bonds within a site (calibrated scores: ~0.76).

SiteMap computes two output scores for each binding site known as the SiteScore (SScore) and the Druggability score (Dscore). Both scores are defined as:

$$\text{SiteScore} = 0.0733n^{1/2} + 0.6688e - 0.20p$$
$$\text{Dscore} = 0.094n^{1/2} + 0.60e - 0.324p$$

Where,

n = number of site points (capped at 100)

e = enclosure score, and

p = hydrophilic score (capped at 1.0).

For SiteScore the hydrophilic score is capped at 1.0 whereas the hydrophilic score is uncapped in Dscore to penalize highly polar sites. This critical feature in Dscore classifies binding sites between “druggable,” “difficult,” and “undruggable” sites on a protein.

RESULTS AND DISCUSSION

A dataset of twenty-four different X-ray crystal structures of GSK-3β was prepared and submitted to SiteMap for druggability assessment, as described in the

methods section. We denote an average of eight surface pockets found on the structure of GSK-3β. Among them, three pockets (1, 4 and 7) were consistently retrieved in all crystal structures analyzed by SiteMap.

Classifying ligand-binding sites of GSK-3β

We first analyzed the SiteScore data to identify plausible ligand-binding sites. Based on the previously recommended cut-off scores, SiteScore can be applied as a classifier to predict ligand-binding sites (SiteScore ≥ 0.80) or non-ligand-binding sites (SiteScore < 0.80) and a score higher than 1.01 indicate highly potential binding sites[26]. Pockets 1 and 7 demonstrate SiteScores higher than 0.80 with promising ligand-binding capabilities, as shown in Table 1. Interestingly, for site 7 the PDB entry 1I09 and 4NM0 reveal two separate pockets very close in space surrounded by the same region. In these cases, the observed low scores were correlated with the small volume of each pocket. In several cases across our dataset, six pockets (2, 3, 4, 5, 6, and 8) have median SiteScores less than the cut-off range.

Classifying druggable binding sites of GSK-3β

In addition to SiteScore, we also analyzed the druggability of each site of GSK-3β (Table 2). Considering the Dscore criteria, binding sites of a protein can be classified into "druggable," "undruggable," and "medium druggable/difficult" sites[26].

Druggable site (Dscore > 0.98)

A typical druggable site is recognized by its good size, deeply buried pocket and often hydrophobic character. Among the eight sites identified, pocket 1 was the largest predicted site with a median Dscore higher than 0.98 (83% cases). Moreover, undruggable sites (Dscore < 0.83) were not identified in any cases for pocket 1, indicating a druggable pocket of GSK-3β (Table 2). All other pockets, in most cases, fail the druggability criteria.

Difficult sites (Dscore between 0.83 and 0.98)

SiteMap druggability scores recognize pocket 7 as a "difficult" site with the highest predicted cases scoring between 0.83 and 0.98. In addition, in few cases pocket 1 (16%), pocket 2 (5%), pocket 3 (18%), and pocket 4 (13%) scored in the intermediate range. Despite the adequate size and volume of the site, with exceptional hydrophilicity, an important reason to classify site 7 as an "intermediate site" is the low hydrophobic nature of the site (quantitative median phobic score is approximately 0.3) (Table 3). To facilitate comparison, we visualized the surface maps of a "druggable" site (pocket 1) and a "difficult" site (pocket 7) identified by SiteMap (Figure 2 and 3). A clear difference is observed for pocket 7,

which lacks sufficient sized hydrophobic regions (yellow maps). The hydrogen-bond donor and acceptor regions of this pocket (blue and red maps, respectively) are scattered over the entire cavity while these regions are more concentrated surrounding the entire hydrophobic region of pocket 1.

Furthermore, we explored the quantitative pocket characteristics of site 7, as summarized in Table 3. This site is relatively open to solvent with moderately high average exposure scores of 0.69 (calibrated score: ~0.49). Moreover, the site is partially buried with an average enclosure score of 0.64 (calibrated score: ~0.78; higher scores are considered better for a deeply buried pocket). The degree of contact measures the relative tightness between a ligand and the binding site. Here, the contact property displayed relatively lower scores, observed to be 0.8 compared to the standard values (calibrated score: ~1). As a result, it would be more challenging to design high affinity drug-like molecules for this site. Moreover, the donor/acceptor character of this pocket quantifies moderate hydrogen-bond possibilities between a well-structured ligand and the site. These features represent the overall characteristics of a "difficult" pocket assessed by SiteMap.

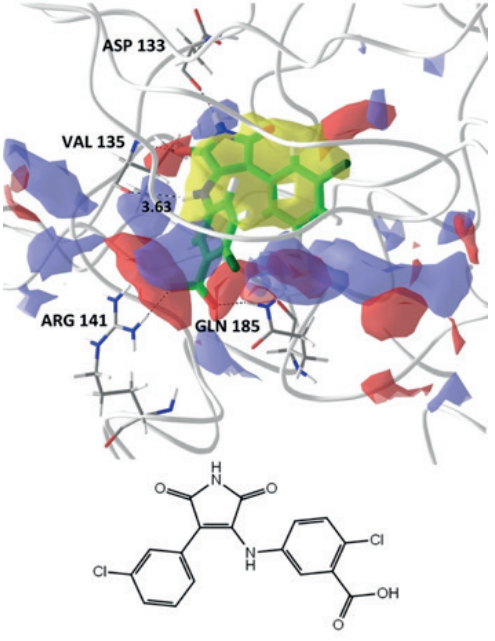

Figure 2. Hydrophobic (yellow), hydrogen-bond donor (blue), and acceptor maps (red) of site 1 (druggable pocket) with co-crystallized ligand 3-anilino-4-arylmaleimide along with a two dimensional (2D) structure representation. (PDB ID: 1Q4L represented as a thin gray tube). For clarity, site points were removed over the entire binding pocket.

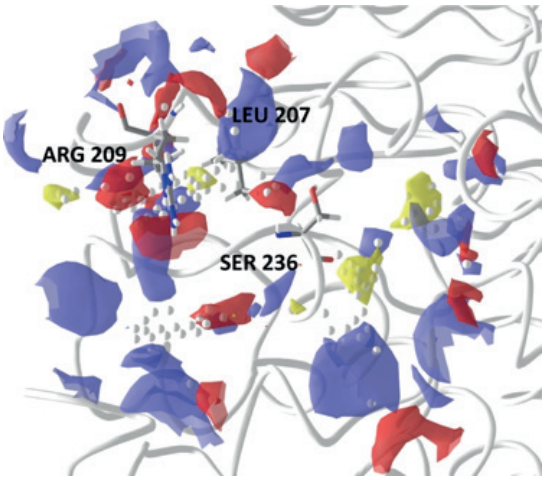

Figure 3. Hydrophobic (yellow), hydrogen-bond donor (blue), and acceptor maps (red) of site 7 (difficult pocket) identified by SiteMap. (PDB ID: 1PYX represented as a thin gray tube and the site points as white spheres).

(iii) Undruggable sites (Dscore < 0.83)

The undruggable sites are shallow protein surface pockets, extremely hydrophilic with negligible hydrophobic nature, characterized by Dscores below 0.80. The druggability scores recognize pockets 5, 6, and 8 as undruggable sites (100% cases). Several cases for pocket 2 (95%) and pocket 4 (87%) were also categorized as undruggable sites (Table 2). SiteMap identified pocket 3 as having two or three distinct cavities within the same binding region, annotated with their respective scores shown in Tables 1 and 2. This yielded a total of 33 sites, of which 27 sites exhibited median Dscores, less than 0.83 (82% undruggable cases).

Table 1. Performance in classifying binding sites of glycogen synthase kinase-3β based on SiteScore.

PDB code	Pocket 1	Pocket 2	Pocket 3	Pocket 4	Pocket 5	Pocket 6	Pocket 7	Pocket 8
1GNG	0.891	0.523	0.726, 0.690, 0.553	0.553	n.f.	0.553	0.963	0.743
1H8F	1.012	0.525	n.f.	0.720	n.f.	0.722	0.937	0.666
1I09	1.035	n.f.	n.f.	0.757	0.703	0.640	0.734, 0.674	0.712
1O9U	0.991	n.f.	0.856, 0.639	0.718	n.f.	0.631, 0.592	0.968	0.722
1PYX	1.029	0.552	n.f.	0.684	0.636	n.f.	0.934	0.697
1Q3D	0.923	0.717	0.566	0.793	0.690	n.f.	0.938	n.f.
1Q3W	1.070	n.f.	0.574	0.775	0.652	0.661	0.944	n.f.
1Q41	1.097	0.742	n.f.	0.680	0.670	0.581	0.967	n.f.
1Q4L	1.045	0.788	0.588	0.860	0.621	n.f.	0.912	n.f.
1Q5K	1.014	n.f.	0.659, 0.561	0.681	0.659	0.494	0.914	0.709
1R0E	1.033	n.f.	0.773, 0.568	0.805	0.608	0.570	0.946	0.711
1UV5	1.043	0.701, 0.605	0.748, 0.621	0.730	0.713	0.823	0.973	0.726
2O5K	1.031	0.804	0.709	0.680	n.f.	0.745	0.969	0.664
2OW3	1.035	0.636	0.836, 0.623	0.919	n.f.	n.f.	0.957	0.772
3DU8	1.058	0.628, 0.627	0.862, 0.686	0.746	n.f.	n.f.	0.947	n.f.
3F88	1.118	0.822	0.691, 0.637	0.940	n.f.	0.479	0.870	0.636
3GB2	1.046	0.584	n.f.	0.661	n.f.	0.484	0.945	0.631
3PUP	1.038	0.597	0.795	0.816	0.631	0.596	0.899	n.f.
3ZRM	1.016	0.575	0.728, 0.700, 0.570	0.645	n.f.	n.f.	0.962	0.649
4ACD	1.033	0.674	0.686	0.742	0.644	n.f.	0.962	n.f.
4J1R	0.930	0.709	0.845	0.627	n.f.	0.636	0.954	0.668
4J71	1.034	n.f.	0.664	0.681	0.724	n.f.	0.994	0.664
4NM0	0.996	n.f.	0.867, 0.626	0.736	0.718	n.f.	0.754, 0.680	0.744
4NU1	1.070	0.930	0.813, 0.651, 0.635	0.735	0.632	0.627	0.810	0.766
Site Score ≥ 0.80	24	03	06	05	00	01	22	00
Site Score < 0.80	00	16	27	19	14	15	04	17
Site not found	none	07	05	none	10	09	none	07

n.f. denotes cavity was not found.

Table 2. Performance in classifying binding sites of GSK-3β based on Dscore.

PDB code	Pocket 1	Pocket 2	Pocket 3	Pocket 4	Pocket 5	Pocket 6	Pocket 7	Pocket 8
1GNG	0.868	0.430	0.680, 0.678, 0.362	0.461	n.f.	0.484	0.974	0.720
1H8F	1.002	0.342	n.f.	0.665	n.f.	0.638	0.943	0.615
1I09	1.005	n.f.	n.f.	0.713	0.575	0.596	0.661, 0.620	0.678
1O9U	1.013	n.f.	0.904, 0.418	0.590	n.f.	0.568, 0.549	0.994	0.641
1PYX	0.987	0.488	n.f.	0.612	0.567	n.f.	0.953	0.671
1Q3D	0.896	0.576	0.487	0.769	0.647	n.f.	0.953	n.f.
1Q3W	1.054	n.f.	0.505	0.732	0.623	0.655	0.960	n.f.
1Q41	1.114	0.723	n.f.	0.622	0.643	0.532	0.999	n.f.
1Q4L	1.068	0.752	0.548	0.868	0.542	n.f.	0.930	n.f.
1Q5K	1.023	n.f.	0.649, 0.516	0.619	0.602	0.410	0.924	0.233
1R0E	1.057	n.f.	0.809, 0.516	0.742	0.564	0.381	0.957	0.653
1UV5	0.969	0.516, 0.553	0.747, 0.570	0.670	0.652	0.776	0.936	0.691
2O5K	1.003	0.690	0.588	0.552	n.f.	0.732	0.979	0.628
2OW3	1.073	0.599	0.851, 0.466	0.935	n.f.	n.f.	0.976	0.721
3DU8	1.058	0.571, 0.573	0.894, 0.673	0.725	n.f.	n.f.	0.946	n.f.
3F88	1.133	0.800	0.555, 0.604	0.989	n.f.	0.359	0.874	0.573
3GB2	1.053	0.520	n.f.	0.603	n.f.	0.405	0.968	0.595
3PUP	1.065	0.475	0.820	0.787	0.574	0.560	0.904	n.f.
3ZRM	1.026	0.454	0.696, 0.686, 0.537	0.596	n.f.	n.f.	0.986	0.598
4ACD	1.038	0.624	0.590	0.696	0.601	n.f.	0.983	n.f.
4J1R	0.933	0.566	0.854	0.579	n.f.	0.509	0.982	0.617
4J71	1.057	n.f.	0.543	0.637	0.691	n.f.	1.031	0.598
4NM0	0.982	n.f.	0.928, 0.496	0.715	0.650	n.f.	0.728, 0.510	0.728
4NU1	1.083	0.882	0.857, 0.469, 0.561	0.703	0.573	0.499	0.730	0.731
Dscore > 0.98 (druggable)	20	00	00	00	00	00	06	00
Dscore (0.83–0.98) (difficult)	04	01	06	03	00	00	15	00
Dscore < 0.83 (undruggable)	00	18	27	21	14	16	05	17

n.f. denotes cavity was not found.

Table 3. SiteMap property values of pocket 7 (Allosteric site) of GSK-3β.

PDB code	Size	Volume	Exposure	Enclosure	Contact	Phobic	Philic	Balance	Donor/ acceptor ratio
1GNG	111	312.816	0.671	0.643	0.799	0.280	1.079	0.260	1.154
1H8F	94	284.690	0.717	0.637	0.808	0.286	1.076	0.265	1.034
1I09	50	143.717	0.717	0.621	0.859	0.179	1.154	0.155	0.771
	33	128.968	0.783	0.652	0.810	0.338	0.953	0.355	1.185
1O9U	105	320.362	0.693	0.643	0.803	0.359	1.017	0.353	1.093
1PYX	91	279.888	0.709	0.638	0.812	0.353	0.001	0.353	0.969
1Q3D	95	277.830	0.716	0.633	0.824	0.252	1.052	0.240	1.123
1Q3W	92	260.337	0.690	0.655	0.880	0.395	1.026	0.385	0.922
1Q41	119	298.753	0.654	0.630	0.820	0.377	0.978	0.386	0.835
1Q4L	84	290.864	0.728	0.631	0.823	0.443	0.951	0.466	1.069
1Q5K	86	290.178	0.730	0.643	0.798	0.410	1.021	0.402	1.220
1R0E	97	300.468	0.731	0.634	0.819	0.327	1.071	0.305	1.145
1UV5	111	312.473	0.683	0.658	0.875	0.131	1.222	0.108	0.780
2O5K	120	303.555	0.636	0.652	0.844	0.476	1.081	0.440	0.832
2OW3	116	319.333	0.707	0.634	0.818	0.231	1.055	0.219	1.315
3DU8	93	286.405	0.677	0.658	0.864	0.351	1.090	0.322	1.114
3F88	77	287.091	0.779	0.626	0.738	0.140	1.000	0.140	1.128
3GB2	97	257.250	0.709	0.626	0.805	0.347	1.022	0.340	1.191
3PUP	84	223.979	0.679	0.638	0.790	0.269	1.046	0.257	1.427
3ZRM	102	301.154	0.690	0.640	0.801	0.459	1.038	0.443	1.026
4ACD	108	297.038	0.667	0.641	0.828	0.424	1.047	0.405	1.122
4J1R	112	286.748	0.652	0.623	0.803	0.370	1.017	0.364	1.065
4J71	104	281.260	0.633	0.659	0.859	0.536	0.935	0.573	1.220
4NM0	40	133.427	0.688	0.671	0.818	0.727	0.826	0.879	1.293
	38	84.721	0.568	0.639	0.849	0.000	1.389	0.000	1.227
4NU1	59	186.249	0.704	0.668	0.856	0.117	1.205	0.097	0.619
Average value	89	259.598	0.693	0.642	0.823	0.330	1.014	0.327	1.072

Phobic and philic terms are the hydrophobic and hydrophilic scores, respectively.

Computational Validation of Generated Pockets

The pockets identified by SiteMap analysis were validated with the known co-crystal structures available at the time of the study. Seven different cavities are supported by X-ray crystallographic studies in the Protein Data Bank (PDB) where ligands (pocket 1), peptides (pocket 2 and 3) and even heteroatoms (pocket 4, 5, 7, and 8) are known to be captured within these cavities, while pocket 6

represents an orphan site.

Pocket 1: A variety of heterocyclic ligands are known to mimic the GSK-3β active site. To validate this site, the PDB structure of 1Q4L was selected which has an anilino-maleimide crystallized within GSK-3β[27]. Quesada-Romero *et al.* reported the orientation of several maleimide derivatives that adopt the GSK-3β active site[28]. Figure 2 has been taken from the same perspective to confirm the druggability and binding of such ligands to this pocket. A maleimide core structure attached to two aryl rings occupies the yellow hydrophobic region. The carboxylate group and one of the acyl groups of the ligand lie in the red acceptor region, while the NH group of the core maleimide structure occupies the blue donor region. The anilino group just failed to spot the donor region. Here, SiteMap accuracy is judged as the distance between the anilino group and the carbonyl oxygen atom of Val135 is 3.63 Å, which is quiet far for a strong hydrogen-bond interaction.

Pocket 2: This pocket is recognized as the substrate binding site. A pS9 auto-inhibitory peptide[29] recognizing the substrate site is shown in figure 4. Key hydrogen-bond interactions are recognized with the primed phosphate groups of the peptide and a triad of three basic residues (Arg96, Arg180, and Lys205). Furthermore, the backbone of the peptide is shown to interact with the Lys94 residue of this site. These interactions confirm the binding of such peptides in this pocket.

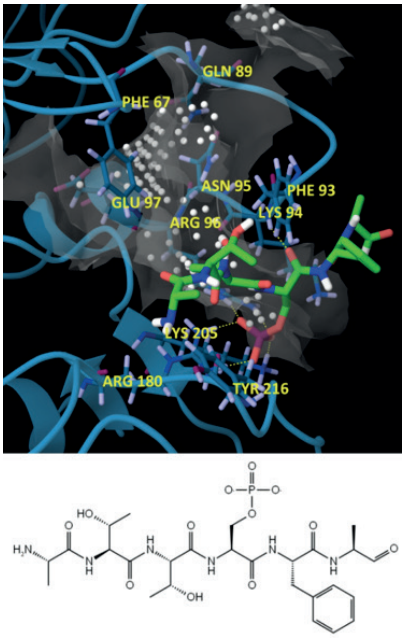

Figure 4. A pS9 auto-inhibitory peptide is shown to interact with the substrate site of GSK-3β in the 4NU1 structure together with a 2D structure representation.

Pocket 3: The Axin and Fratide peptides recognize the peptide-binding channel comprising α-helix (residues 262-273) and the extended loop (residues 285-299) at the C-lobe of GSK-3β[30–32]. In several cases across our dataset, two to three small cavities were observed within the entire channel. Among these, a distinct "hydrophobic patch" identified by SiteMap formed with the hydrophobic residues of the α-helix, and the extended loop provides favorable peptide-binding characteristics (Figure 5).

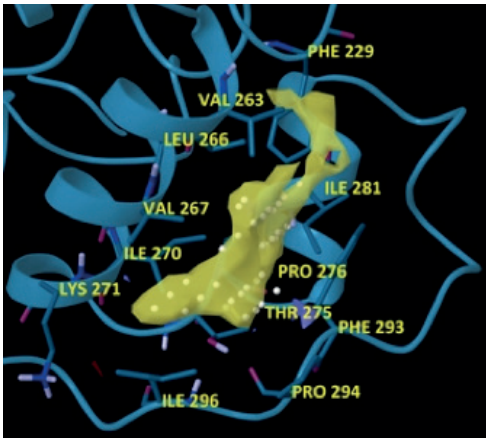

Figure 5. Pocket 3 identified as a hydrophobic patch by SiteMap within the peptide-binding channel at the C-lobe of GSK-3β.

Key hydrogen-bond interactions are recognized with residues Tyr288 and Glu290 and the Fratide peptide (Figure 6B) and with the Asp264 residue and the Axin peptide (Figure 6A).

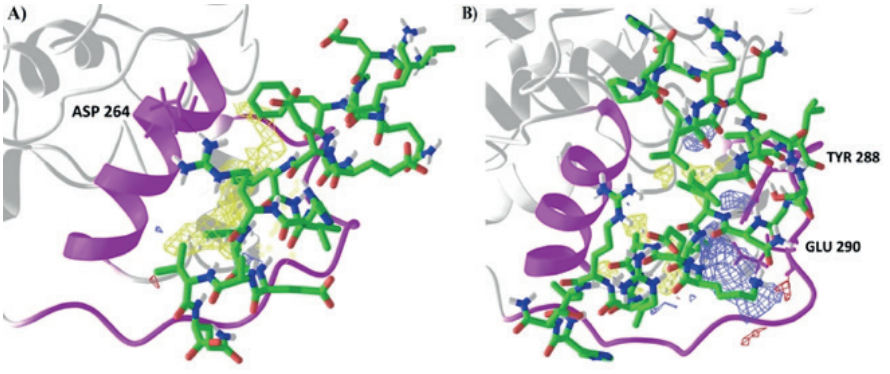

Figure 6. A) Axin peptide represented as green tube recognize the peptide-binding channel (purple cartoon representation) with key H-bond interaction represented as black dots (PDB ID: 1O9U). **B)** Fratide peptide represented as green tube recognize the peptide-binding channel with key H-bond interactions represented as black dots (PDB ID: 1GNG).

Pocket 4: This pocket was visible in each case analyzed by SiteMap. The cavity generated by SiteMap and the presence of glycerol with a hydrogen bond to the Arg144 residue is observed in the PDB structure of 4NU1 shown in Figure 7. Key interactions are observed between the oxygen atom of the glycerol molecule and the guanidine NH group of Arg144.

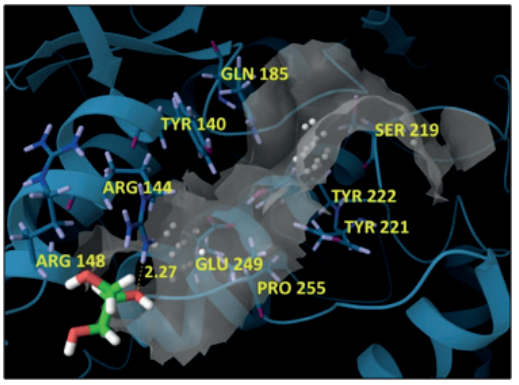

Figure 7. A glycerol molecule represented as a green tube is shown to capture the surface of pocket 4 (gray) with the key H-bond highlighted as yellow dots (PDB ID: 4NU1, blue cartoon representation).

Pocket 5: A shallow pocket is located at the N-lobe of GSK-3β. In the PDB structure of 4NU1, a molecule of glycerol is shown to bind in this pocket. The polar and charged residues (Tyr56, Lys86, and Asn129) surrounding the small cavity are significant enough to form hydrogen-bond contacts with small hydrophilic glycerol molecules, as shown in Figure 8.

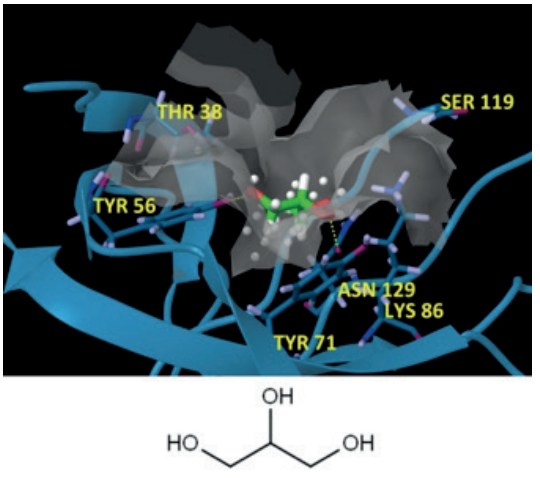

Figure 8. A glycerol molecule represented as a green tube together with a 2D structure is shown to capture the surface of pocket 5 (gray) with the key H-bond highlighted as yellow dots (PDB ID: 4NU1, blue cartoon representation).

Pocket 6: The major residues lining a small cavity in the hinge domain were identified as pocket 6 (Figure 9). No ligands or heteroatoms are known to bind in this pocket, which represents an orphan site.

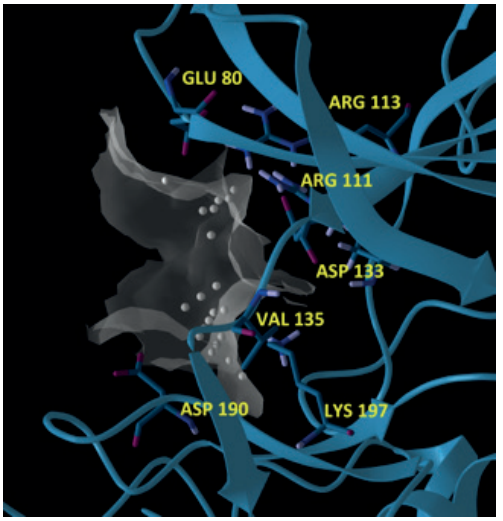

Figure 9. The total surface of pocket 6 represented as gray and the site points as white spheres (PDB ID: 1O9U, blue cartoon representation).

Pocket 7: SiteMap scores recognize pocket 7 as the most promising allosteric site of GSK-3β. In the crystallization experiments, a few heteroatom and reagent molecules are known to be captured in this pocket. To support our result, the PDB structure of 4NM0 was selected, which has Dithiothreitol (DTT) molecule crystallized within GSK-3β[29]. Key hydrogen-bond interaction is recognized between the DTT molecule and residues Thr326, Ala327, and Arg319 of pocket 7, as shown in Figure 10. In addition, the binding residues Arg209 and His173 of the same pocket in the PDB structures of 1UV5, 1I09, and 1GNG also chelates phosphate and sulfate ions present in the crystallization experiments. These interactions are crucial for understanding the important residues for allosteric modulation of the kinase.

Pocket 8: A new small pocket identified by SiteMap is located at the C-lobe of GSK-3β. The presence of glycerol with a hydrogen-bond to Val155 is observed in the PDB structures of 3ZRM (Figure 11). The presence of such hydrophilic molecules can provide clues for the prevalence of these pockets on GSK-3β.

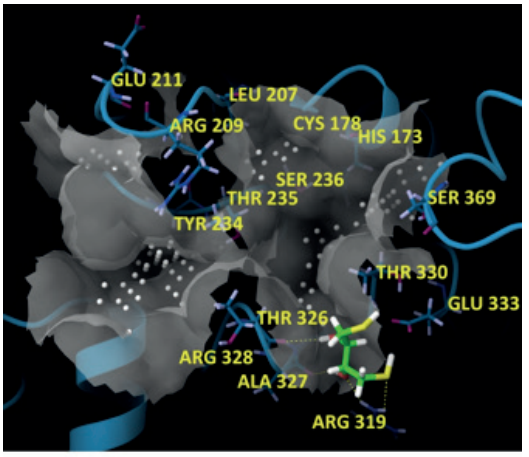

Figure 10. A Dithiothreitol (DTT) molecule represented as a green tube together with a 2D structure is shown to capture the surface of pocket 7 (gray), with the key H-bond highlighted as yellow dots (PDB ID: 4NM0, blue cartoon representation).

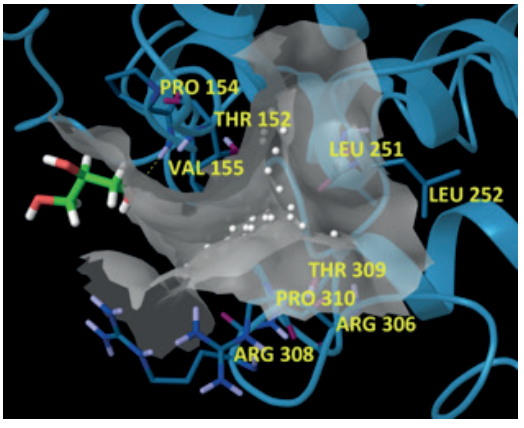

Figure 11. A glycerol molecule represented as a green tube is shown to capture the surface of pocket 8 (gray) with the key H-bond highlighted as yellow dots (PDB ID: 3ZRM, blue cartoon representation).

CONCLUSIONS

Predicted druggability on the pockets of GSK-3β were assessed by SiteMap. With the aim to identify druggable sites of GSK-3β, SiteMap studies yielded useful insights that clearly distinguish druggable, difficult, and undruggable sites. These results conclude that the ATP-binding site is the only druggable pocket of GSK-

3β, while pocket 7 is a "difficult" pocket, as analyzed by SiteMap. Although this pocket is classified as a "difficult site" by SiteMap analysis, it has earmarks as a good binding site and the possibility of designing selective allosteric modulators. With the help of SiteScore we successfully differentiated the ligand and non-ligand-binding sites of GSK-3β; however, the identification of non-ligand-binding sites or difficult/undruggable sites does not mean that a ligand cannot bind to such sites. Moreover, it would be challenging to search such drug-like ligands that bind with high affinity on these sites. At the moment, SiteMap studies have classified the pockets of GSK-3β based on the druggability score, which can clearly classify these sites between druggable, difficult, and undruggable sites. We predict that these results will add to the accuracy in identifying druggable pockets of GSK-3β.

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CONFLICT OF INTEREST

None declared.

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Applications of Cell Culture Studies in Pharmaceutical Technology

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ABSTRACT

There have been advances in the cell culture models for research and drug studies. The cytotoxicity and permeability of drug molecules and delivery systems are evaluated by cell culture models both in the pharmaceutical industry and in academia. Cell models serve as an important platform to investigate cytotoxicity and permeability studies by reducing the use of animal models. Since 3D cell models mimic in vivo cells better, it plays a significant role in the testing of drugs. This review article emphasizes an overview of cytotoxicity and permeability studies and 3D cell culture model used in pharmaceutical technology.

Key words: cell culture, cytotoxicity, permeability, pharmaceutical technology

INTRODUCTION

Studies conducted during the initial development of drugs such as toxicity, corrosion and drug activity were carried out on animals; however, in the past 10 to 20 years, alternatives have been sought due to the fact that animals do not effectively model human in vivo conditions and unexpected responses are observed in the studies. Cell culture studies made positive contributions to the initial development of drugs. Contrary to animal studies, the need for low drug and a short response time are the characteristics for in vitro cell culture methods[1]. In 2005, more than 100 million animals were used and 10 billion dollars were spent for animal toxicity experiments[2]. It is possible to reduce this cost and the amount of animal use for experiments with well-designed cell culture studies[3].

Among other health authorities, the FDA, has recommended the use of the human cell line to identify metabolic pathways for drugs and shared their applicability in in vitro tests in guidelines published in 2004[4]. In November 2013, the

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National Institutes of Health reported that more than 230 embryonic stem cell lines are appropriate for research[5].

The number of publications on cytotoxicity and permeability studies using cell cultures as seen on PUBMED clearly shows that these topics have been trending higher over the past several years (Figure 1).

Figure 1. Numbers of publications in Pubmed using keywords 'cell culture', 'cytotoxicity' and 'permeability'.

CANCER CELL LINES AS MODEL FOR DRUG STUDIES

Cancer cell lines are used as a model for research and drug studies (Table 1). During the development of therapies, drugs are tested and developed by using cancer cell lines as an important model[6]. Drugs are tested on cancer cell lines by pharmaceutical companies[7].

Table 1. Most used cancer cell lines (modified from Ferreira et al., 2013[8]).

Cancer cell line	Species	Disease
HeLa	*Homo sapiens*	Cervix adenocarcinoma
Caco-2	*Homo sapiens*	Colorectal adenocarcinoma
MCF-7	*Homo sapiens*	Breast adenocarcinoma
A549	*Homo sapiens*	Human lung carcinoma
U87MG	*Homo sapiens*	Glioblastoma-astrocytoma
HT-29	*Homo sapiens*	Colon adenocarcinoma
HEP-G2	*Homo sapiens*	Hepatocellular carcinoma
K-562	*Homo sapiens*	Chronic myeloid leukaemia
Cos7	*Cercopithecus aethiops*	SV40 transformed - kidney
PC3	*Homo sapiens*	Prostate adenocarcinoma
A375	*Homo sapiens*	Malignant melanoma
HEK 293	*Homo sapiens*	Human Embryonic Kidney 293 cells
CHO	*Chinese hamster*	Chinese hamster ovary cell line

The use of cancer cell lines for drug studies has advantages and disadvantages. Advantages of cancer cell lines are: They can be easily handled and manipulated. They have high homogeneity. Cancer cell lines have similarity with the initial tumour and it makes them advantageous to test cancer drugs on cancer cell lines. For experiments, they are unlimited auto-replication source and are easily substituted. The experiment results of the cancer cell lines for drug studies are reproducible. They also have disadvantages, such as they can be cross-contaminated with Hela cells. During studies, they can lose homogeneity and genomic stability, and they are also susceptible to contamination with bacteria and mycoplasma. And also, another difficulty is that the growth of long-term cancer cell lines is challenging[8].

Drug Screening in Cancer Cell Lines

Drug development begins with drug testing in cancer cell lines. Afterwards, drugs can be tried in in vivo clinical trials. Researchers have evaluated cytotoxicity of drugs on cancer cell lines for many years and data from these experiments have been proven to have clinical predictive value[9,10]. Diverse responses to drugs are displayed by different cancer cell lines[9]. Cell line panels are also useful for drug tests. The first cancer cell line is the panel NCI-60 which utilizes 60 cancer cell lines. This cancer cell line panel was developed to reduce animal experiments for testing of the drugs[11]. The mechanism, physiological processes and treatments of diseases can be explored by the help of the release of molecules from drug delivery carriers, drug diffusion tests and drug toxicity tests. Drug efficacy tests and drug toxicology tests are valuable as they present an alternative to animal experiments[6,12]. REACH is a regulation of European Union with four phases named registration, evaluation, authorization and restriction of chemicals and indicates that animal testing on chemicals should be avoided. 7th Amendment to the Cosmetics Directive of European Union declares that finished cosmetic products and ingredients should be tested on alternative non-animal tests. This regulation of European Union has made cell-based experiments even more important[13].

In Vitro Systems for Toxicity Testing

Cell culture conditions are improved to mimic more closely an in vivo growth environment. These improvements are co-culture with normal cells such as myofibroblasts and immune cells and three-dimensional (3D) matrices. Levels of specific growth factors and additives can be controlled by microfluidic perfusion systems[14].

Toxicity testing of new drugs will eventually be done in animal models to under-

stand overall toxicity. If toxicity testing of new drugs is done in appropriate in vitro cell lines, limited animal model toxicity tests are needed. Toxicity tests on cell lines can be evaluated by testing the drug on a variety of cell types for which cell lines are available. These toxicity tests on cell lines can be an indication for the drug treatments for the cancer type which was studied[14,15].

Although a suitable source of in vitro normal cell cultures were needed, only cancer cell lines were a suitable source for these tests. Normal cells from human-induced pluripotent stem cells (hiPSC) and improved epithelial cell culture conditions can be used nowadays to broaden in vitro toxicity testing at the normal cellular level[14,15]. hiPSC-derived cardiomyocytes, endothelial cells, hepatocytes, and neuronal cells are commercially available. In the future, liver and heart cells will be available through these techniques to be tested and toxicity testing in whole animals may be reduced[14].

Cell Viability Assays

Cell viability assays are widely used for in vitro drug and formulation toxicology studies. There are alternative assays for cell viability[16,17,18]. Commonly used assays are for cytotoxicity or cell viability detection: the MTT assay, the LDH assay, the neutral red, XTT assay and AlamarBlue assay. Activity of lactate dehydrogenase in the extracellular medium is measured by the LDH assay. Cell death is indicated by intracellular LDH release into the culture medium[19]. The neutral red assay also indicates cell viability. The neutral red is taken into the cell by living cells and sequestrated in the lysosomes of cells[20,21]. The MTT assay is a cell viability assay which determines cytotoxicity, and the validity of this assay was determined in cell lines[22]. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt. The tetrazolium ring of tetrazolium salt within the mitochondria is cleaved by succinate dehydrogenase, which results in its conversion to an insoluble purple formazan. The insoluble purple formazan accumulates in healthy cells due to the impermeability of the purple formazan to the cell membranes[23]. MTT or XTT are tetrazolium salt reduced to a colored formazan according to viable cell number[16,17]. Tetrazolium salts (MTT or XTT) are reduced by generation of NADH and NADPH. These colored formazans are measured in an automated colorimeter. MTT assay has an extra solubilization step for formazans which has to be dissolved in dimethylsulfoxide (DMSO) before colorimetric measurement. XTT tetrazolium assay was developed to eliminate this solubilization step and viable cells directly metabolize XTT reagent to a water soluble formazan[16,24]. Optical density in the culture wells can be directly read by calorimetry. Use of Alamar Blue as a fluorescent dye for cell viability tests started in 1993[25]. Alamar Blue is a non-fluorescent, non-toxic blue dye which is

reduced to a pink fluorescent dye as a result of cell viability[26].

Cell-based in vitro models have been used to study drug permeability through buccal (TR146 cell culture), intestinal (Caco-2 cell, TC7, MDCK, LLC-PK1), nasal (cultured nasal cells), pulmonary (Calu-3), ocular (corneal epithelial cells), rectal, vaginal (cervical cell lines) routes[27]. Among these, intestinal permeability is the most studied because the oral route of drug administriation is the most common[28].

Intestinal Permeability by Cell Culture

Genomics, proteomics, robotics and in silico chemistry are used to reduce costs in the drug discovery cycle. Understanding biopharmaceutical properties such as solubility, metabolic stability and intestinal permeability is an important task in the industry[29]. The physico-chemical properties of the active drug substance and its product, the physiological functions of body tissues and organs, and the complex process of drug absorption are influenced by physical and biochemical properties of the epithelial barrier[30]. While permeability of drug compounds through the intestinal membrane is a complex process[29]. The mechanism of drug transport in cell cultures is by passive transcellular and paracellular transport and active-carrier mediate transport[31]. The intestinal permeability of a drug can be evaluated by many techniques[29]. These methods are: 1) in vitro tissue methods (using diffusion chamber); 2) in vitro cell methods- (Caco-2; MDCK); 3) in vitro artificial membranes (parallel artificial membrane permeability assay (PAMPA) or immobilized artificial membrane (IAM) columns); 4) in vivo methods (whole animal pharmacokinetic studies; 5) in situ methods (single-pass perfusion); 6) computational approaches. These methods for the permeability assessment of drugs can be used individually or in combination. Cell culture models are preferred for the permeability assays due to predictability and throughput [29]. Recent studies for cell culture models for pharmaceutical technology are shown in Table 2.

Human cell culture models for the toxicity test of aerosolized nanoparticles are Alveolar epithelial cells (A549) and Airway epithelial cells (Calu-3, 16HBE140-, BEAS-2B)[32]. As nanosized ZnO are used in sunscreens, the effect of the cytotoxicity of ZnO is important. Two commercially available ZnO powders' cytotoxicity was tested in human colon-derived RKO cells[33]. Albanese and Chan, 2011 produced transferin-coated gold particles by Frens method and they tested the effect of aggregation of particles on three different cell lines (HeLA, A549, MDA-MD-435) due to the fact that aggregation of particles can affect toxicity. They concluded that uptake of particles are more affected than toxicity[34]. In another study, the effects of different sized silver nanoparticles on cytotoxicity were eval-

Table 2. Examples of recent studies for pharmaceutical technology

Studies	Cell line used toxicity testing /permeability test
In vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung—A dialog between aerosol science and biology[32]	co-cultures of two different epithelial cell lines, A549 and 16HBE14o- epithelia cell lines
ZnO particulate matter requires cell contact for toxicity in human colon cancer cells[33]	RKO colon cancer cells
Effect of gold nanoparticle aggregation on cell uptake and toxicity[34]	HeLa and MDAMB-435 cells
Impact of silver nanoparticles on human cells: effect of particle size[35]	A549, SGC-7901, HepG2 and MCF-7 cells
Cellular uptake and toxicity of gold nanoparticles in prostate cancer cells: a comparative study of rods and spheres[36]	PC-3 cells
Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells[37]	IP15 (glomerular mesangial) and HK-2 (epithelial proximal) cell lines
Thermoreversible Pluronic® F127-based hydrogel containing liposomes for the controlled delivery of paclitaxel: in vitro drug release, cell cytotoxicity, and uptake studies[38]	KB cancer cells
Cytotoxic effects of iron oxide nanoparticles and implications for safety in cell labelling[39]	C17.2 neural progenitor cells, PC12 rat pheochromocytoma cells and human blood outgrowth endothelial cells
Cytotoxicity induced by engineered silver nanocrystallites is dependent on surface coatings and cell types[40]	Mouse macrophage (RAW-264.7) and lung epithelial (C-10) cell lines
Vitamin E TPGS coated liposomes enhanced cellular uptake and cytotoxicity of docetaxel in brain cancer cells[41]	C6 glioma cells
Curcumin loaded poly (2-hydroxyethyl methacrylate) nanoparticles from gelled ionic liquid–In vitro cytotoxicity and anti-cancer activity in SKOV-3 cells[42]	SKOV-3 ovarian cancer cell lines
Toxicity of copper oxide nanoparticles in lung epithelial cells exposed at the air-liquid interface compared with in vivo assessment.[43]	HBEC or A549 cells.
Cytotoxicity assessment of lipid-based self-emulsifying drug delivery system with Caco-2 cell model: Cremophor EL as the surfactant[44]	Caco-2 cells
Impact of lipid-based drug delivery systems on the transport and uptake of insulin across Caco-2 cell monolayers[45]	Caco-2 cells
Cellular uptake and transcytosis of lipid-based nanoparticles across the intestinal barrier: relevance for oral drug delivery[46]	Caco-2 cells
Regional Morphology and Transport of PAMAM Dendrimers Across Isolated Rat Intestinal Tissue[47]	Caco-2 cells

uated on four human cell models (A549, SGC-7901, HepG2 and MCF-7). The experiments with 5 nm, 20 nm and 50 nm silver nanoparticles showed that the smallest - (5 nm) is the most toxic nanoparticle among them. They concluded that the reason may be that when nanoparticles are smaller, they enter cells more easily than larger ones[35]. Properties of gold nanoparticles make them an important tool for cancer therapy, gene delivery and cancer detection. Gold nanoparticles of various types (plain spherical, PEGylated spherical and PEGylated rods) were compared with each other to evaluate cytotoxicity on a human prostate cancer cell line (PC-3 cells). The results showed that the cytotoxicities of these gold nanoparticles were not different from one another[36].

Metallic nanoparticles are used in medical treatments but can have a toxic effect on the kidneys. Pujalté et al., 2011 tested nanoparticles (TiO_2, ZnO and CdS) which were produced for industry on human renal culture cells. While TiO_2 nanoparticles showed no cytotoxicity, ZnO nanoparticles showed dose-dependent cytotoxicity and CdS nanoparticles are the most toxic[37].

In another study, Paclitaxel loaded liposomes were incorporated into a thermoreversible hydrogel called Pluronic F127 in order to improve the solubility of paclitaxel and increase drug loading. Human oral cancer KB cell lines were incubated with PTX formulation loaded with liposomal 18% F127 gel, Taxol or liposome. Blank liposomal F127 gel was found to be safer than pure liposome[38].

Iron oxide nanoparticles are used for cell labelling in biomedical research. 4 different types of iron oxide nanoparticles were produced and their toxicity was tested on human blood outgrowth endothelial cells, C17.2 neural progenitor cells, and PC12 rat pheochromocytoma cells. Non-toxic concentration was determined for these nanoparticles to be used for the MR visualization[39].

Silver nanoparticles were used for their antimicrobial properties and biomedical applications such as wound dressings. Different silver nanoparticles were produced. Toxicity measurements were performed on lung epithelial (C-10) cell lines and mouse macrophage (RAW-264.7). In this study, they concluded that surface charge and coating materials used in the synthesis, particle aggregation, and the cell-type used for the tests affect the cytotoxicity results. Based on cytotoxicity results, macrophage cells were found to be more sensitive than lung epithelial cells[40].

In one study, liposomes coated with a PEGylated vitamin E (TPGS) with docetaxel were developed for treatment of brain tumours. Cytotoxicity of the liposomes were tested on C6 glioma cells. TPGS coated liposomes have higher cytotoxicity than PEG coated liposomes [41].

The anticancer activity of Curcumin loaded poly (2-hydroxyethyl methacrylate) nanoparticles was tested on ovarian cancer cells (SKOV-3) and the results showed that Curcumin loaded poly (2-hydroxyethyl methacrylate) nanoparticles exhibited a better level of tumor cells regression activity than free curcumin[42].

In another study, an evaluation of the toxicity of copper oxide nanoparticles was performed on lung adenocarcinoma cells (A549 cells) and human bronchial epithelial cells (HBEC) using an *in vitro* air–liquid interface (ALI) exposure system. Exposure of CuONP significantly reduced cell viability in a dose-dependent manner. CuONP were more toxic on A549 cells than HBEC[43].

Lipid-based self-emulsifying drug delivery systems are used for solubilizing poorly soluble drugs. When excipients and formulations are toxic, they damage cell monolayers and this artificially increases drug permeation. Understanding their toxicity is important for the correct interpretation of results. Bu et al., 2016 showed that using Cremophor EL as the surfactant did not damage the Caco-2 cell layer and did not induce toxicity in the lipid-based self-emulsifying drug delivery system[44].

In one study, Self-(nano)-emulsifying drug delivery systems (SNEDDSs) containing insulin were produced to transport insulin across the intestinal membrane. Size of SNEDDS were between 35-50 nm. They demonstrated that two SNEDDS formulations increased the permability of insulin in Caco-2 cell monolayers[45]. In another study, permeability of nanostructured lipid carriers and solid lipid nanoparticles were compared and validated by Caco-2 cell monolayers. Permeability results of nanostructured lipid carriers were higher than solid lipid carriers[46].

Intestinal permeability of Polyamidoamine (PAMAM) dendrimers were compared to Caco-2 monolayers and isolated rat intestinal regional mucosae. TEER values of Caco-2 monolayers and isolated rat intestinal regional mucosae matched each other[47].

Cell Culture Model for Drug Permeability Studies

Absorption of drugs mostly occurs in the small intestinal region of the gastrointestinal tract. The small intestine selectively absorbs major nutrients, digests foreign substances and is a barrier to digestive enzymes. The surface of the small intestinal region increases the potential surface area available for digestion and absorption[29]. Models of human intestinal epithelium have been developed. This culture model is an ideal system to test the intestinal permeability of drug candidates. The Caco-2 cell is the model cell line which has been studied the most, characterized and is most useful for drug permeability studies[48,49,50] (Figure 2).

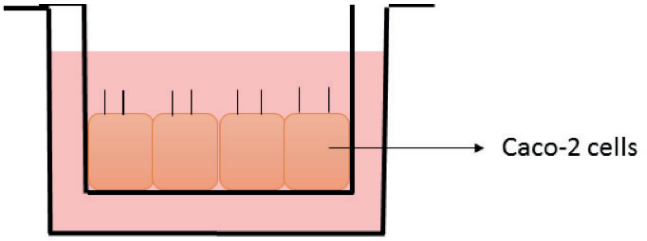

Figure 2. Caco-2 permeability assay.

Caco-2 is a human colon adenocarcinoma cell line[48]. It has well-established tight junctions and undergoes spontaneous enterocytic differentiation. Caco-2 is also used to predict the oral absorption of drugs in humans due to similarity of the Caco-2 cell line's permeation characteristics of drugs with permeation characteristics of human intestinal mucosa. Use of Caco-2 cells as a screening tool is becoming more widespread in the pharmaceutical industry. Lewis lung carcinoma-porcine kidney 1 (LLC-PK1) cells and Madin–Darby canine kidney (MDCK) are other cell line models used in permeability studies. Also, the 2/4/A1 line, transfected cells and Caco-2 cell clones are modified cell models which can be used for permeability studies. MDCK cell line is obtained from dog kidney cells. MDCK cell line is used as a model for intestinal permeability and use of this cell line as a model was first discussed in 1989[51]. Since then, MDCK cells were used for the permeability studies of early drug discovery compounds. Differentiation into columnar epithelial cells, formation of tight junctions and epithelial cell characteristics are common properties of Caco-2 and MDCK cells[52]. Permeation of passively absorbed drugs in Caco-2 cells and MDCK cells were correlated with each other.While Caco-2 cells grow in three weeks, MDCK cells grow in three days and it makes MDCK cells advantageous for the shorter cultivation period. As cell contamination and labour are disadvantage of longer cell culture time of Caco-2, shorter cultivation time of MDCK cells becomes important. The disadvantages of MDCK cells versus Caco-2 are: permeability values of drugs may be different for transporter-mediated uptake and/or efflux compounds due to species difference[53]. LLC-PK1 cells are also alternative cell line to Caco-2 for permeability studies and this porcine cell line can be utilized for the passive absorption of drugs[54]. The 2/4/A1 line is obtained from fetal rat intestine and passive paracellular permeability of 2/4/A1 line is similar to human small intestine. In vitro permeability models were improved by these modified cell lines for carrier mediated transport[55,56].

Challenges Associated with Cell Culture Models

The use of cell culture models in permeability studies presents some issues; these are: Important transporters for drugs are expressed in Caco-2 cells[57]; however, expression of transporters in Caco-2 is lower than human small and large intes-

tine[58]. Lower expression of these transporters may yield to less correlated results between Caco-2 cells and human intestine. Gene expression profiles of solute carrier transporters (SLC), efflux transporters (ABC) and cytochrome P-450 enzymes are different among Caco-2, MDCK and human intestine[60]. These differences may affect the permeability of compounds which are specific to transporters showing different expression profiles[57]. TEER values and permeability values of Caco-2 from different laboratories can be different due to varying culture conditions[60,61,62]. Although the transepithelial electrical resistance (TEER) of small intestine is estimated to be in the range of 25–40 Ω cm^2, TEER of Caco-2 cells are 234 Ω cm^2. Pore sizes of intestinal epithelium and Caco-2 cells are 5 Å and 6 Å, repectively[63]. When Caco-2 and MDCK cell lines were transfected with influx transporters, proper permeability results similar to human intestine were achieved. Cytochrome P450 (CYP3A4) is oxidative CYP enzyme in intestine and it is less expressed in Caco-2 cells compared to human intestine[64]. Caco-2 cells can have different permeability values compared to human intestine due to cacophilicity, which means a drug reversibly binds to Caco-2 and it results in underestimation of permeability values. Although pH of human intestine varies from acidic to slightly basic pH[65], Caco-2 grows in fixed pH conditions. After cell-based permeability studies, sample analysis is mostly done using LC-MS tools. However, high content of salt in the transport buffer affects LC-MS by interfering with ionization[29].

3D CELL CULTURE MODELS

Cell-based screening has been revolutionized by 3D cell culture technologies. While in 2D cell culture cells were grown on flat surfaces, in 3D cell culture cells were grown with the help of attachment surfaces such as extracted extracellular matrix (ECM)[66]. The ECM is the complex mixture of proteins and sugars beyond the membrane of the cell[67]. Collagen, laminin and glycosaminoglycans, such as chondroitin sulfate and heparan sulfate are widely used components of ECM[68,69]. The basement membrane is a specialization of the ECM required for adhesion of the epithelial cell layer and responsible for a wide range of epithelial cell phenomena including cell identity, wound healing and migration[69]. ECM is not just a random mix of secreted components, but a specific composition of biochemicals and defined geometrical structure, which stimulates specific cell responses, such as differentiation[70]. Filter well inserts, sponges and gels and microcarriers are types of ECM. Filter well inserts are the first technology used for ECM[66]. 2D cell culture tests, animal model tests and clinical trials are the processes for drug discovery. Drugs may fail during phase III due to the toxicity of the drugs or efficacy of the drugs[71,72]. Drug test failures on 2D cell cultures led to the development of 3D cell cultures as an improved model for testing. Results of the drugs' responses

are different between 2D and 3D cultures due to differences between 2D culture and human intestine. Understanding the toxicity of the new drug before animal tests is important in minimizing costs during research and development[73,74]. Cellular responses, spatial organization of cell surface receptors, gene expression and cellular behaviour in 3D culture cells can differ from 2D cell culture and it can be concluded that 3D-cultured cells reflects in vivo cellular responses better than 2D cell culture[75]. 3D cell cultures are grown using a scaffold or in a scaffold-free manner[76]. In 2D cell culture, cells form a monolayer while they are growing on a flat surface; attached cells proliferate. When they die, they detach from the surface and these dead cells are removed during the medium change[77]. 2D cells are flatter than in vivo cells. This dissimilar morphology affects the characteristics of the cells and they do not properly mimic the behaviours of the cells in the body. However, 2D cell culture is still the commonly used in vitro test in drug screening[76].

In 3D cell culture, cell–cell interactions and cell–ECM interactions can provide the in vivo environment easily. 3D cell culture contains cells that are in various stages. Viable cells are at the outer part of the cluster, the core part contains cells at hypoxic state due to deficiency of medium[78]. The relative proliferation between 3D and 2D-cultured cells showed different trends and this proliferation rate difference also depends on cell line and matrix[79,80,81,82]. 3D cell culture has different gene, protein, and cell receptor expression compared to 2D cell culture[75]. Susewind et al., 2016 developed a 3D intestinal model by embedding human macrophages (THP-1) and human dendritic cells (MUTZ-3) in a collagen cell and seeding Caco-2 cells on top of them. Non-inflamed and inflamed co-cultures were used to understand inflammation effects and cytotoxicity of nanoparticles. Comparison of Caco-2 monocultures and 3D co-cultures showed that cytotoxicity and interleukin release, which is an important biomarker for inflammation, was higher in 3D co-cultures[83]. Gomez-Roman et al., 2017 tested temozolomide, bevacizumab and erlotinib on 2D and 3D Glioblastoma cultures. They proved that these three drugs affect 2D and 3D cultures and 3D model responses were similar to clinical trials[84]. In a recent study by Ribas et al., 2016, the authors created a vascular microenvironment of the heart for drug development[85]. In another recent study by Marsono et al., 2016, human iPSC-derived cardiomyocytes were used to stimulate 3D construction of myocardium[86].

CONCLUSION AND FUTURE PROSPECTS

Cell culture is increasingly used in pharmaceutical research and regulators support the use of cell culture during the drug development stage. This review summarizes basic techniques of human cell line studies in the pharmaceutical tech-

nology field. Cell-based experiments are highly predictive for preclinical drug toxicity and permeability assessments. It is assumed that, in the future, animal and human clinical trials will be greatly reduced by 3D cell culture experiments.

AUTHOR CONTRIBUTIONS

These authors contributed equally.

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Possible Health Risk of Electromagnetic Fields

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ABSTRACT

Electromagnetic field (EMF) is a kind of radiation and is emitted to environment from some medical diagnostic equipment, radio and television, communication devices such as cell phones, and other electrical appliances. It can have some risks to biological systems. There are some published documents on the possible human health risks of it including epidemiologic, *in vivo*, and *in vitro* studies. This review article focuses on the advers health effects of electromagnetic fields on reproductive and developmental system, psychological system, nervous system, genotoxic effects, carcinogenesis, ear and vestibular system, ocular system, melatonin production and circadian rhythms. Although there are some studies which indicated some possible effects on these physiological systems, the data is limited to reach exact conclusion. Further studies are needed to prove safety and biological effects of EMF.

Key words: Electromagnetic field, electromagnetic radiation, health risks

INTRODUCTION

Electromagnetic fields (EMFs)

Electromagnetic fields (EMFs) are emitted to environment from radio and television, communication devices such as cell phones, and other electrical appliances at homes and workplaces. EMF composed of waves of electric and magnetic energy moving together through space. It is a type of radiation which is emitted by all cell phones. Different types of electromagnetic energy are categorized by their wavelengths and frequencies and comprise the electromagnetic spectrum. Different technologies use different radiation frequency. During recent years, people exposed to EMF radiation with widespread use of cell phones or their

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base station. This station could affect people's health[1,2]. Electromagnetic radiation (EMR) is radiated from the electrically charged particles and also called electromagnetic wave (EMW). They travel through air and in other substances[3].

Classification of EMWs

EMWs can be classified as ionizing and non-ionizing radiation according to their frequency and energy. Non-ionizing radiation refers to any type of EMR that does not carry enough energy to remove an electron from an atom or a molecule. Sources of non-ionizing radiation include microwaves, radio waves, cordless phones, wireless networks (Wi-Fi), power lines and magnetic resonance imaging (MRIs). Ionizing radiation has high-frequency waves with enough energy to release electrons from molecules. It can damage the structure of cells in the body (including DNA). It has well-documented effects on human health. Ionizing radiation is emitted by radon, uranium, and other naturally occurring radioactive elements and is used for X-rays, nuclear medicine, and CT ("cat") scans[3].

Specific Absorption Rate (SAR)

When an organism is exposed to EMFs, it absorbs energy. The amount of absorbed energy depends on many factors such as the frequency of the radiation, the power density, the electrical properties of the exposed tissues and the orientation and possible attenuation of the fields. Exposure to radiofrequency (RF) energy is determined by the SAR, a measure of the rate at which energy is absorbed by the body when exposed to RF. It is defined as the power absorbed per mass of tissue, measured in watts per kilogram (W/kg)[4].

The SAR is commonly used to measure the power absorbed during MRI scans and from cell phones. The allowable SAR limit for the head is 2 W/kg[4].

Health Risks of EMFs

Previous studies reported that low-level exposure to EMF radiation could cause a wide range of health effects, including behavioral changes, effects on the reproductive system effects, changes in hormone levels, headaches, and cardiovascular effects.

Reproduction and Developmental Disorders

Infertility is worldwide problem. It is affected by not only medical problem but also a psychological stress, including anxiety, depression and problems in maintaining the marital relationship. Chromosomal abnormalities, micro deletions, cystic fibrosis, transmembrane conductance regulator mutations, genetic factors, environmental factor are major causes of infertility. Also life style like smoking and drinking may partake to infertility. Besides, radio and television

which transmit radiofrequency electromagnetic waves (RF EMWs) with frequency ranging from 0.5 MHz in the Amplitude Modulation (AM) radio band up to 30,000 MHz in radar band can have some effects on the fertility. In addition, hair dryers, X-ray equipment, laboratory equipment (incubators, centrifuge), computers and cell phones are EMF producers[5].

Male infertility disorders

It is important to investigate the effect of EMR on male fertility. A possible link between EMR emitted from cell phone and infertility were seen in several studies[5,6].

Exposure to radiofrequency electromagnetic radiation (RF EMR) and mild scrotal heating can induce DNA damage in mammalian spermatozoa, although the underlying mechanisms are unclear. The induction of DNA damage in spermatozoa has been associated with male infertility, early pregnancy loss and morbidity in the offspring, including childhood cancer.

EMF released by cell phone can have adverse effects on human fertilization potential. Various preliminary studies, though with limitations, have suggested a use-dependent decrease in seminal quality and testicular tissue damage in men using cell phones. However, the mode of this damage to male reproductive system by EMF is still unclear. At high intensities, EMF may cause reversible disruption of spermatogenesis because of heating properties of RF radiations lead to thermal effects. High-quality research is still needed in this field[6].

Wang et. al. reported that Leydig cells injury may affect spermatogenesis on mice[7]. These cells are among the most susceptible cells in to EMF. On the other hand, 890-915 MHz EMF exposure to rats for 20 min per day for 1 month resulted as no effects on the testis of rats[8].

The first human study was designed to evaluate the infertility problem on men, the duration of cell phone use was correlated negatively with the proportion of rapidly progressive motile spermatozoa. The prolonged use of cell phone might have negative effects on the sperm motility[9].

Another experimental study was done to determine the biological and morphological effects of 900 MHz EMF on male rat testes for 4 weeks. The results revealed a decrease in seminiferous tubular diameter and epithelium thickness without effect on spermatogenesis after applying of 900 MHz. There was also a significant decrease in serum total testosterone level and not a statistically significant decrease in luteinizing hormone and follicle stimulating hormone levels[10].

Significant damage to mitochondrial and nuclear genome was observed in a study conducted on epididymal spermatozoa of mice with 900 MHz EMF for 7 days and 12 h a day [11].

An another study was designed on semen samples of 27 healthy men exposed to 900 MHz cell phone at distance of 10 cm for 5 min. Significant decrease in rapid progressive motility, increase in slow progressive motility and increase in the percentage of immotile spermatozoa were observed in that study[12].

The effect of cellular phone use on the fertility of males subjected to marital infertility therapy was assessed by Wdoviak et.al. A decrease in the percentage of live sperm cells in a vital, progressive motility in semen is correlated with the frequency of use of cell phones. An increase in the percentage of sperm cells with abnormal morphology is associated with the duration of exposure to the waves emitted by GSM equipment[13].

The use of cell phones adversely affected the quality of semen by decreasing the sperm counts, motility, viability and morphology in related to daily active cell phone use and talking time of man[14].

To see microscopic changes in the seminiferous tubules, male rats were exposed to EMF (1835 to 1850 MHz) from cell phones. Each cage was provided with 8 cell phone sets in active silent mode. These cell phones were kept in a small metal cage with a wooden bottom in order to address concerns that the effects of exposure to the phones could be due to heat emitted by phones rather than to RF EMW alone. These small cages which were then placed in the plastic cages of rats. It was resulted histological and morphological effects of testes. This investigation showed that number of seminiferous tubules were decreased significantly in exposed subgroups day by day after 110 day. At the same time diameter of seminiferous tubules were decreased[5].

In contrast to possible adverse effect of EMF, the main bio functional sperm parameters in healthy men exposed to the different use of the cell phone were investigated. The results showed that none of the conventional sperm parameters examined were significantly altered. The trousers users showed a higher percentage of sperm DNA fragmentation compared to other groups[15].

Female sexual function and fertility disorders

There is limited data on the association between EMF exposure during pregnancy and reproductive outcomes. Some studies have reported increased risk of spontaneous abortions and congenital malformations[16].

The effect of EMF on early development of chick embryos was investigated. EMF

exposed groups were influenced the mortality ratio when the exposure duration and the power level was increased. High frequency electromagnetic field can be responsible for the alterations in growth and development *in ovo* amniotic vertebrates[17].

EMF emitted by Wi-Fi (2.45 GHz) and cell phone (900 and 1800 MHz) were studied to evaluate the effects on oxidative stress and trace element levels in the kidney and testes of growing rats from pregnancy to 6 weeks of age. Lipid peroxidation and oxidizable iron content increased and antioxidant trace elements (copper and zinc), and glutathione levels decreased during kidney and testis development[18].

Psychological Disorders

Babadi-Akashe et. al. studied the behavior of cell phone addicts and mental health of university students. They found that the rates of students' addiction to cell phones reduced in related to increased and improved mental health[19].

Nervous System Disorders

Narayanan et. al studied the brain effects of the EMF emitted from the cell phones in rat and the quite substantial hazard on passive avoidance behavior and hippocampal morphology in rats was detected[20]. EMF from cellular phones did not lead to anxiety or not cause impairment of the working memory, but it may cause stressful behavior pattern in rats[21]. Decreased immobility and increased locomotor activity were seen in rats after exposure to EMF[22]. The spatial learning and memory function of mice were affected after exposure to EMF[33].

Fragopoulou et. al. found out no significant differences in the spatial memory test, and morphological assessment of the brain of rats after EMF exposure. However, in some exposed animals, there were decreased locomotor activity, increased grooming and tendency of increased basal corticosterone levels. These findings suggested that EMF exposure may lead to abnormal brain functioning[24].

The possible link between cellular telephone use and risks for various diseases of the CNS such as Alzheimer's disease, migraine, or vertigo was investigated. It was found a weak, but a statistically significant association between cell phone use and migraine and vertigo[25].

The blood brain barrier (BBB) damage

The human brain is surrounded by a layer of specialized cells which act as a barrier between brain and the contents of bloodstream. This barrier prevents toxins from reaching brain and maintains a healthy environment for brain tissue. Nittby et. al. studied the effect of EMF on BBB permeability in rats. It was found that the EMF exposure reduced memory functions[26].

Genotoxic Effects/Cell Damage

The potential of RF EMF to cause changes in a cell's genetic material (DNA) and/or to damage the genome is an important research area. Genotoxic substances can potentially cause mutations or cellular damage that can contribute to the development of malign tumors. It was reported a positive correlation among EMF exposure from mobile phones and DNA damage, chromosomal aberration, increased sister chromatide exchange frequencies in humans[27,28,29].

Ferreira et. al. investigated the effects of ultra high frequency-electromagnetic field (UHF-EMF) on micronucleus formation in erythrocytes and imbalances in free radical metabolism in liver and blood of rat offspring whose mothers were irradiated during the pregnancy. The study was resulted that UHF-EMF was indeed able to generate cell chromosome damage transplacentally. However the effects of free radical metabolism on this damage was not clear[30].

Gandhi and Singh reported an increased micronucleated buccal cells and cytological abnormalities in cultured lymphocytes of individuals exposed to EMF from mobile phones[31,32].

EMF induced DNA single-strand and double-strand breaks in human diploid fibroblasts and in rat granulosa cells in culture were also reported[33].

Carcinogenic Effects

Several studies showed that long-term exposures to EMF caused an increase in the risk of some types of tumors, but experimental studies were not available to explain the exact association. Overall, the epidemiological studies on the possible relationship between EMF exposure from mobile communicating devices and cancer have been conducted. Most of these studies have focused on brain tumors. Some have found a risk of cancer with long-term exposure while others have not.

Due to head exposure to EMF released from mobile communicating devices, studies mainly focused on the relationship between EMF exposure and brain tumors[34].

International Agency for Research on Cancer (IARCH) coordinated a study in 1998 and 1999. They concluded that the relationship between cell phone use and brain tumor risk would be feasible and informative[35].

Holding a cell phone to the ear can result in high SAR values in the brain depending on the EMF released from mobile communicating devices, their holding places in the body and the quality of the link between the base station and device. Cancer risk is limited for two types of brain tumor: glioma and neuroma. Although there was some statistically significant evidence about the association

between EMF from cell phone use and brain tumor risks, most studies showed no association[36].

Long term epidemiological studies for an association between long-term cell phone use and the risk of brain tumor showed a link between prolonged cell phone use and ipsilateral brain tumor, but the observed effect was not extended to meningiomas[37,38].

Hardel et. al. evaluated the risk of brain tumors with long-term use of cell phone. The result showed that long-term use (10 years or more) of cell phones was associated with increased risk of brain tumors (ipsilateral glioma and acoustic neuroma). However, no association was found for contralateral tumours[39].

Lagario et. al. assessed the intracranial tumors and cell phone use and they reported the occurrence of intracranial tumors. In addition, they stated the combined relative risk in long-term cell phone users (≥ 10 years) for meningioma[40].

Relationship between glioma and use of cell phones and cordless phones was assessed by Hardel et.al. The risk of glioma with use of cell phones in the >25 year latency group and increased risk was observed with the use of cordless phones in the >15-20 year[41].

On the other hand there were some studies showing no association between brain tumors and cell phone use, but a possible association was estabilished between heavy cell phone use and brain tumors[42]. The study conducted to evaluate the risk of malignant melanoma in the head and neck regions was resulted no association on long term cell phone use of more than 10 years and total wireless use[43].

Although there were some studies on the risk of brain tumor, there were limited data about the other cancer risks. Paulsen et. al. conducted a cohort study to evaluate skin cancer risk and mobile phone use. They reported no correleation[44].

Ear and Vestibular System Disorders

Cell phones are usually held in close proximity to the ear. These exposures could have an adverse effect on hearing function either at the level of inner ear or on the central auditory pathways.

Hutter at.al. reported that high intensity and long duration of cell phone use might be associated with tinnitus[45]. After long-term and intensive cell phone use, inner ear damage and hearing loss may be seen[46,47,48].

The effect of long term cell phone use on auditory brainstem evoked responses was studied and it was resulted in no effect on auditory pathways from cochlear nerve to auditory brainstem[49].

Ocular Effects

Eyes are unprotected by the skull and comprised of cells that are extremely sensitive to electromagnetic energy. The eyes can absorb electromagnetic energy very quickly.

The possible effects of long term use of cell phone on eyes of human was studied. Blurred vision, eye inflammation, lacrimation, redness in the eyes, visual disturbance and increased secretion of the eyes were reported[50].

Effects on Melatonin Production and Circadian Rhythms

Melatonin is a hormone that controls circadian (sleep/wake) rhythms. It is secreted at night by the pineal gland and produces many biological effects. It also reduces risk of neurodegenerative diseases[51].

Burch et. al. investigated the relationship between cellular telephone use and excretion of the melatonin. It was found that prolonged use of cellular telephones might lead to reduced melatonin production. Morover elevated EMR exposures may potentiate the effect[52].

Organ Damage

Oktem et. al. examined 900-MHz cell phone-induced oxidative stress on renal tubular damage and the role of melatonin to protect kidney tissue against oxidative damage in rats. It was found the increase in malondialdehyde levels of renal tissue and N-acetyl-β-D-glucosaminidase in urine and also the decrease in renal superoxide dismutase, catalase, and glutathione peroxidase activities. They demonstrated the role of oxidative stress induced by EMR, and the protective effect of melatonin against oxidative tissue injury in rat kidney[53].

Erdem et. al. studied the effect of continuous exposure to 50 Hz EMF on the levels of trace elements in serum and different organs of Guinea pigs. They reported that Cu and Mg levels were affected in serum and tissue samples of these animals[54].

CONCLUSION

As a conclusion, EMR emitted from different devices including communication technologies such as mobile phones, wireless equipments, television, electrical appliances can have different health risks to human. Users should be careful about the potential health risks and should control them to use of these devices. The time spent with the cellular devices should be reduced as low as possible. If possible speakerphone or a wired headset use could be recommended to reduce the exposure to EMR. The cell phone and other wireless devices should be kept

several feet from the bed. Carrying a cell phone in pants or shirt pocket will emit EMR to nearby tissues. For this reason cell phones should carry away from body parts if it is possible.

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Can Tissue Factor, A Multifactorial Molecule of the Hemostasis, be used As A Biomarker for Thrombosis, Inflammation and Cancer?

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ABSTRACT

Tissue factor (TF) is a transmembrane protein found in many tissues and is active in various biological reactions. It is a member of the cytokine receptor superfamily and is referred to as CD 142 because of this feature. TF is also known as Factor III in the coagulation system and binds FVII/VIIa. The TF and FVIIa complex has both procoagulant and signaling activities. It functions in many biological processes, including hemostasis, thrombosis, inflammation and cancer. TF is essential for haemostasis but increased TF expression within atherosclerotic plaques and TF positive microparticles were detected in thrombotic conditions. TF increases inflammation by enhancing intravascular fibrin deposition and activates protease-activated receptors (PARs). TF and FVIIa complex also contribute to tumor growth by activating PAR2. Recent retrospective studies have shown that TF positive microparticles increase in the plasma of cancer patients. Therefore TF may be suggested to be used as a biomarker. However further studies are required to reveal the availability of TF as a biomarker to identify cancer and risk of thromosis and inflammation.

Keywords: Tissue Factor, Hemostasis, Thrombosis, Inflammation, Cancer

INTRODUCTION

Hemostasis, which allows blood to circulate without clotting, allows bleeding to stop through a rapid response to tissue damage. A clot is formed through reactions involving blood vessels, thrombocytes, coagulation and fibrinolytic system, which then bleeding stops and dissolves the fibrin clot. [1-5]

The work of hemostasis in a vascular damage is summarized as follows: 1-The artery in the region is vasoconstricted, the purpose is to slow the blood flow through the re-

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gion, 2-Circulating thrombocytes recognize endothelial damage, apply adhesion, secretion and aggregation, therefore rapidly forming a weak clot, 3-The coagulation proteins in the circulation recognize that they've encountered an environment different than the endothelium, and intact clots form in the interrupted region, 4-The fibrinolytic system helps repair the injured area, allowing the fibrin to break down and the blood flow in the area to return to normal. In this system, clotting is simultaneously created and prevented. The blood running in a vessel does not coagulate but immediately it coagulates when encounters a foreign surface such as tissue factor and collagen. Endothelial cells play a major role in stopping a bleeding and preventing thrombus formation. [6-11]

The mechanism of partial thromboplastin time (PTT) and prothrombin time (PT) tests used in the follow-up of diagnosis and treatment of bleeding and coagulation-related diseases is clotting when blood encounters non endothelial surface.[12] This mechanism is important for arterial thrombus formation. In summary, it is not wrong to say that the formation of blood clots in endothelial dysfunction is not well managed.

The hemostatic system parameters continue to be at the center of scientific research with unknown aspects, while providing opportunities to facilitate diagnosis and treatment on bleeding and thrombosis events. As in the past, the incidence of thrombosis in arterial and venous circulation is currently very high. Atherosclerotic cardiovascular and cerebrovascular diseases are still major causes of morbidity and mortality worldwide. [13-17]

Tissue factor, which is present as Factor III in the clotting pathway and also known as "CD 142" and "thromboplastin", is a transmembrane protein which enables thrombin formation and is multifunctional. Parallel to today's technology, there are many studies aiming to better know the structure of tissue factor, while reporting that this protein is not only effective in the clotting system, but also in inflammation and cancer formation. [18-33]

The purpose of this article is to draw attention to how some phases of hemostasis developed historically and encourage young researchers to shine a light on the unknown aspects of this subject.

Historical development of and current knowledge on hemostasis

The hypothesis that the German physician Rudolph Virchow, who lived between 1820 and 1902, which corresponds to the thrombosis aspect of present-day hemostasis;

1-Intravenous wall change (thrombosis due to atherosclerotic change, inflammatory change)

2-Change of blood components (hypercoagulability, thrombocyte activation, anticoagulant insufficiency)

3- It is in the form of blood flow alteration (Deep vein thrombosis and pulmonary embolism)

Numerous additions have been made to this hypothesis through scientific studies in parallel with the development of technology, but Wirschoff's thrombus formation hypothesis still remains valid (13,18-13).

Thrombocytes in Hemostasis

1842 is considered as the year of birth for thrombocytes. Because in those years, four different researchers, unaware of each other, reported that a different particle was circulating differently from erythrocytes and leukocytes. In 1846, Zimmerman noticed that these particles formed aggregates. The following rapid developments in these studies have led to a better understanding of the role of thrombocytes in hemostasis. In 1956, Ulutin and Karaca first announced to the world the the mechanisms of secretion of thrombocytes, and that disorders in this mechanism would cause a disease [1-2,34-42]

Coagulation Proteins and Tissue Factor (CD 142)

The foundations of the coagulation mechanism were laid in 1834. In those years, brain tissue suspensions were found to be immediately lethal when administered intravenously to animals. This finding led to the understanding that tissue extracts formed clots in blood. Tissue factor had a place in Schmidt's works in 1892 and in Morawitz's work in 1904 and tissue factor, prothrombin and fibrinogen were all portrayed in the simple mechanism of the coagulation system. After the year 1900, the studies focused on the tissue factor of clotting mechanism for a long time. Works to purify tissue factor started in 1912 with Howell, and in 1944 with Chargaff et al. Studies on this purification of tissue factor continued until the beginning of 1980s. Later studies were rather on the genetics of tissue factor. At the end of the 1980s the tissue factor gene was isolated and cloned. From the 1990s onwards, tissue factor was considered to be a real initiator in the coagulation system. Among 13 clotting proteins, only the tissue factor is an integral membrane protein. Clotting factors exhibit structural homology, while only the tissue factor exhibits homology with type 2 cytokine receptors. For this reason, tissue factor was also named as "CD 142". [18-33, 43]

Tissue factor, a transmembrane protein, is present in various proportions in all tissues, mostly in the brain, lung, and uterus. The tissue factor contains protein, phospholipid and carbohydrate in varying proportions according to the tissue from which it is obtained. The carbohydrate portion of the tissue factor is added

by posttranslational modification. Tissue factor is a cofactor for Factor FVII in cell membranes. It is comprised of 263 amino acids in total. 219 of these amino acids are located in the extracellular region, 23 amino acids in transmembranal regions and 21 amino acids are in intracellular regions. The extracellular region of the tissue factor contains the binding site for Factor VII. [18-33]

In 1947 Owren reported that Factor V was necessary for the formation of the clot and this invention was followed by other coagulation proteins. Concerning the conversion of prothrombin to thrombin, Prof.Dr. Walter H. Seegers' intensive studies and contributions from other researchers led to significant advances in clotting mechanisms between 1905-1950. The identification and mechanism-description of the 13 clotting proteins present in the present coagulation pathway, expressed as "clotting factors", were carried out by Davie and Ratnoff in 1964 (36).The current flow chart of the intractable coagulation system has emerged from the examination of patients with bleeding findings with clotting defects. [13, 43-46]

The Prothrombin time test, administered by Quick in 1935, remains among today's gold standards in the control of the clotting system [4] In our faculty, Medical and Dental Practice students in the 1st grade isolete tissue factor from the bovine lung and use it in the prothrombin time test. The students presented this study in İstanbul Medipol University "Student Scintific Days" in 2015 and received a runner-up prize[47] Figure 1 shows the prothrombin time test's flow diagram.

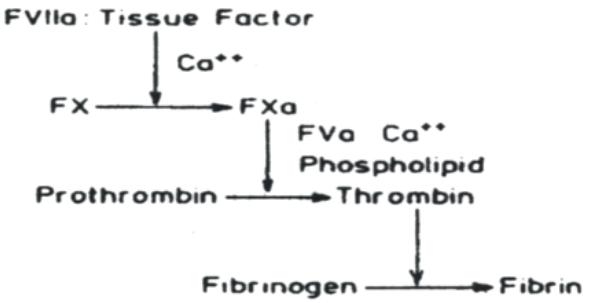

Figure 1. Biochemical reactions take place in the prothrombin time test (Ref.12)

Tissue Factor and Thrombosis

Adverse changes leading to thrombus formation in the blood stream begin with hypercoagulation and endothelial activation. Wirschoff explains the reasons for the thrombus that still remain valid today with the possibilities of the 1800's. One of the most important aspects of the present invention is the demonstration of the presence of microparticles bearing a tissue factor. Dimensions of microparticules carrying TF vary between 30-1000nm. Microparticles are released

from activated leukocytes and thrombocytes. Hatemi[11] reported that in diabetic and especially type 2 diabetic patients, thrombophilia is observed and that these patients have increased coagulation factors, especially fibrinogen, and also increased thrombocyte adhesion and aggregation, while fibrinolytic activities were observed to be in a decrease. Today, contrary to previous knowledge, the presence of biocompatible tissue-factor-bearing microparticles has been demonstrated and it is predicted that the tissue factor may be a biomarker in this area since the importance of arterial thrombus formation is emphasized. [5-17,26,27,31,48-54]

Is Tissue Factor a Proinflammatory Agent?

Endothelial cells are a common point between coagulation and inflammation. Inflammatory diseases are known to increase tendency to thrombus. In 1936, life-threatening thromboembolic events were seen in ulcerative colitis cases, which were thought to be associated with increased tissue factor. Fibrin deposits are seen in synoviums of patients with rheumatoid arthritis. The extracellular portion of the tissue factor obtained with the recombinant technique was injected into the articulations of healthy mice and 80% of the mice developed arthritis. Studies on which factor to remove for the purpose of preventing the relation between inflammation and coagulation have not yet reached a point of clarity; this subject is still yet to be resolved. [32, 33, 55-61]

Tissue Factor and Cancer

Patients with cancer are more likely to have thrombosis. The relationship between thrombosis and cancer first began with the observations of Armand Trousseau in 1865. Various studies have shown that tissue factor expression increases in cancerous cells. In physiological conditions, the amount of micro particles bearing tissue factor is low. These particles are seen to increase in cases of cancer. TF plays an important role in development of physiological and pathological angiogenesis. Since TF-deficient transgenic mice had deteriorating vascular integrity, embryogenic death occurred in a short time and abnormal development of embryonic development was observed. Similar histopathologic results were seen in VEGF-deficient embryos. Here it is understood that TF and VEGF act similarly. Proangiogenic and antiangiogenic factors are essential for vessel growth and development. TF shows its effect on tumor growth, metastasis and angiogenesis independently of the clotting system but dependently on clotting. TF indirectly increases angiogenesis and tumor growth in coagulation-independent mechanisms by forming fibrin directly or via thrombin. In the coagulation-dependent case, it is known that increasing TF expression leads to increased fibrin formation. [28-30, 62-68]

Control in Hemostatic System

Hemostatic system is internally controlled. Minimal thrombin coagulation, which forms out of very small amounts of tissue factor, activates the system by activating proteins and thrombocytes in the coagulation cascade. These effects of thrombin on procoagulants are positive feedback reactions in the system. Thrombin, on the other hand, converts the protein C to the active protein C (formerly autoprotrombin II-A), which is an anticoagulant, with the effect of the protein C receptor (EPCR) and thrombomodulin found in the endothelium. Figure 2 shows positive and negative feedback reactions of the clotting system. That is, the clot formed with minimal tissue factor is neutralized with minimal tissue factor.

Protein C complex, which exhibits anticoagulant activity by inhibiting strong cofactors such as Factor VIII and Factor V in physiological conditions, has not yet been successfully used in therapeutic applications such as heparin. However, both protein C pathway and tissue factor inhibitor pathway (TFPI) are vital in hemostatic control. This anticoagulant, first identified as autoprotrombin II-A by Seegers and colleagues because of its relevance to prothrombin, has then been referred to as "protein C". Emekli and Ulutin reported that protein C inhibited fibrin production in disseminated intravascular coagulation induced rabbits and the animal model used in this study was the first in the literature. [69-84]

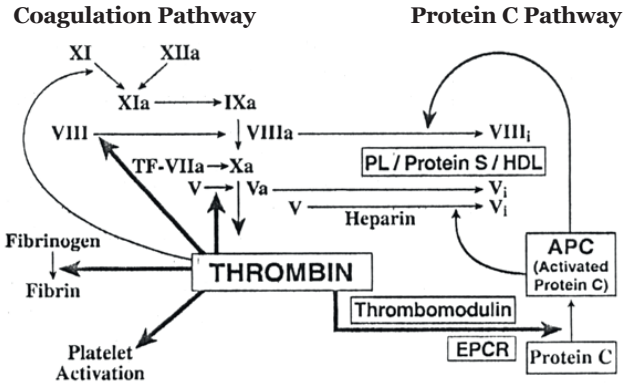

Figure 2: Effect of thrombin on anticoagulant system driven by procoagulant and protein C in the clotting mechanism (Ref.84)

Heparin is a heterogeneous polysaccharide with glucose derivatives and sulphates in its structure. It has been researched since 1800s and is still used as an anticoagulant in treatments. [85-86]

Prof. Dr. Kasım Cemal Güven, who founded Acta Pharmaceutica Sciencia in

1953 and maintained it throughout the years, demanded this Journal be published by İstanbul Medipol University from this year onwards. [87-90]

In conclusion, vascular, thrombocyte, coagulation and fibrinolytic systems of hemostasis are a complex reaction sequence containing negative-positive feedback reactions, multienzymes systems, humoral and cellular procoagulant and anticoagulants. Tissue factor (CD 142), which is included in this system and has versatile functions, is a current topic in today's studies in that it can be a biomarker for arterial-venous thrombus, inflammation and cancer.

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