

Факултет Фармацеутски

УНИВЕРЗИТЕТ У БЕОГРАДУ

01 број 2257/1-1
(Број захтева)Већу научних области медицинских наука
(Назив већа научне области коме се захтев упућује)10.09.2026.
(Датум)**ЗАХТЕВ**

за давање сагласности на одлуке о усвајању извештаја Комисије за оцену докторске дисертације и о именовању комисије за одбрану

Молимо да, сходно члану 47. ст. 5. тач. 4. Статута Универзитета у Београду ("Гласник Универзитета", број 186/15-пречишћени текст и 189/16), дате сагласност на одлуку о усвајању извештаја Комисије за оцену докторске дисертације:

КАНДИДАТ **МИЛАН (ЈОВИЦА) ЈОВАНОВИЋ**
(име, име једног од родитеља и презиме)

студент докторских студија на студијском програму ДОКТОРСКЕ АКАДЕМСКЕ СТУДИЈЕ, ФАРМАЦЕУТСКЕ НАУКЕ, ФАРМАЦЕУТСКА ХЕМИЈА

пријавио је докторску дисертацију под називом:

„Хеминформатичка анализа, дизајн и испитивање инхибитора липидних и протеин киназа као потенцијално нових антинеопластика“

из научне области: ФАРМАЦЕУТСКА ХЕМИЈА

Универзитет је дана 11.10.2022.године својим актом под бр. 02-01 број 61206-3695/2-22 дао сагласност на предлог теме докторске дисертације која је гласила:

„Хеминформатичка анализа, дизајн и испитивање инхибитора липидних и протеин киназа као потенцијално нових антинеопластика“

Име и презиме ментора : 1. Др сц. Катарина Николић, редовни професор, Универзитет у Београду – Фармацеутски факултет (Ужа научна област: Фармацеутска-медицинска хемија и структурна анализа)
2. Др сц. Милкица Цревар, ванредни професор, Универзитет у Београду – Фармацеутски факултет (Ужа научна област : Фармацеутска-медицинска хемија и структурна анализа)

Комисија за оцену докторске дисертације именована је на седници одржаној 18.06.2026.године одлуком факултета под бр. 01 бр.1458/2 , у саставу:

Име и презиме члана комисије	звање	научна област	Установа у којој је запослен
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1. Др сц. Бранка Ивковић, ванредни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармацеутска-медицинска хемија и структурна анализа), председник
2. Др сц. Мара Алексић, редовни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Физичка хемија)
3. Др сц. Славица Ољачић, ванредни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармацеутска-медицинска хемија и структурна анализа)
4. Др сц. Марија Поповић Николић, виши научни сарадник, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармација –Фармацеутска хемија)
5. Др сц. Бранислав Станковић, виши научни сарадник, Универзитет у Београду – Институт за нуклеарне науке „Винча“ (Ужа научна област: Физичка хемија)

Напомена: уколико је члан Комисије у пензији навести датум пензионисања.

Датум стављања извештаја Комисије и докторске дисертације на увид јавности: 09.07.2026.године.

Наставно-научно веће факултета усвојило је извештај Комисије за оцену докторске дисертације наследници одржаној дана 10.09.2026.године.

Комисија за одбрану докторске дисертације именована је на седници одржаној 18.06.2026.године

одлуком факултета под бр. 01 број 1458/2, у саставу:

Име и презиме члана комисије	звање	научна област	Установа у којој је запослен
1. <u>Др сц. Бранка Ивковић, ванредни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармацеутска-медицинска хемија и структурна анализа), председник</u>			
2. <u>Др сц. Мара Алексић, редовни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Физичка хемија)</u>			
3. <u>Др сц. Славица Ољачић, ванредни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармацеутска-медицинска хемија и структурна анализа)</u>			
4. <u>Др сц. Марија Поповић Николић, виши научни сарадник, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармација –Фармацеутска хемија)</u>			
5. <u>Др сц. Бранислав Станковић, виши научни сарадник, Универзитет у Београду – Институт за нуклеарне науке „Винча“ (Ужа научна област: Физичка хемија)</u>			

Напомена: уколико је члан Комисије у пензији навести датум пензионисања.

ДЕКАН ФАКУЛТЕТА

- Прилози:
1. Одлука Наставно-научног већа о усвајању извештаја Комисије за оцену докторске дисертације и одлука о именовању Комисије за одбрану докторске дисертације
 2. Извештај Комисије о оцени докторске дисертације
 3. Примедбе на извештај Комисије о оцени докторске дисертације (уколико их је било) и мишљење Комисије о примедбама

Напомена: Факултет доставља Универзитету захтев са прилозима у електронској форми и у једном писаном примерку за архиву Универзитета

УНИВЕРЗИТЕТ У БЕОГРАДУ
ФАРМАЦЕУТСКИ ФАКУЛТЕТ
11000 - БЕОГРАД
Ул. Војводе Степе 450.
01. број 2257/1
10.09.2026. године

На основу члана 28. Статута и предлога Комисије за последипломске студије – докторске студије, Наставно-научно веће Универзитета у Београду – Фармацеутског факултета на седници одржаној 10.09.2026.године, донело је

О Д Л У К У

ПРИХВАТА СЕ позитиван извештај Комисије за оцену и одбрану завршене докторске дисертације, кандидата **маг.фармације Јовановић Ј. Милана** под насловом: **„Хеминформатичка анализа, дизајн и испитивање инхибитора липидних и протеин киназа као потенцијално нових антинеопластика“** и упућује Већу научних области медицинских наука на усвајање, а по добијеној писаној сагласности одобрава јавна одбрана пред Комисијом у саставу:

1. Др сц. Бранка Ивковић, ванредни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармацеутска-медицинска хемија и структурна анализа), председник
2. Др сц. Мара Алексић, редовни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Физичка хемија)
3. Др сц. Славица Ољачић, ванредни професор, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармацеутска-медицинска хемија и структурна анализа)
4. Др сц. Марија Поповић Николић, виши научни сарадник, Универзитет у Београду - Фармацеутски факултет (Ужа научна област: Фармација – Фармацеутска хемија)
5. Др сц. Бранислав Станковић, виши научни сарадник, Универзитет у Београду – Институт за нуклеарне науке „Винча“ (Ужа научна област: Физичка хемија)

Универзитет је дана 11.10.2022.године својим актом бр.: 02-01 бр: 61206-3695/2-22 дао сагласност на предлог теме докторске дисертације.

Кандидат маг. фарм. Милан Јовановић, објавио је резултате из ове докторске дисертације у два рада категорије М21 у међународним часописима са СЦИ листе:

1. **Jovanović, M.; Nikolić, K.; Čarapić, M.; Aleksić, M.M.** Electrochemical and Theoretical Study on Interaction between Erlotinib and DNA. *J. Pharm. Biomed. Anal.* 2023, 234, 115560, doi:10.1016/j.jpba.2023.115560.
IF2021 = 3,571; Chemistry, Analytical (24/87); M21
2. **Jovanović, M.; Radan, M.; Čarapić, M.; Filipović, N.; Nikolić, K.; Crevar, M.** Application of Parallel Artificial Membrane Permeability Assay Technique and

Chemometric Modeling for Blood–Brain Barrier Permeability Prediction of Protein Kinase Inhibitors. *Future Med. Chem.* 2024, 16, 873–885, doi:10.4155/fmc-2023-0390.
IF2022 = 4,2; Chemistry, Medicinal (16/63); M21

Одлуку доставити: кандидату, Универзитету, члановима комисије, декану, секретару, продекану за последипломске студије, Одсеку за наставу и студентска питања, менторима (Проф. др Катарина Николић и Проф. др Милкица Цревар), Одсеку за правне и опште послове, пословном секретару, председнику Комисије за последипломске студије – докторске студије (Проф. др Биљана Антонијевић) и архиви.

**ПРЕДСЕДНИК
НАСТАВНО-НАУЧНОГ ВЕЋА
ФАРМАЦЕУТСКОГ
ФАКУЛТЕТА**

**Проф. др Наташа Богавац
Станојевић**

НАСТАВНО-НАУЧНОМ ВЕЋУ
УНИВЕРЗИТЕТА У БЕОГРАДУ – ФАРМАЦЕУТСКОГ ФАКУЛТЕТА

КОМИСИЈИ ЗА ПОСЛЕДИПЛОМСКЕ СТУДИЈЕ – ДОКТОРСКЕ АКАДЕМСКЕ
СТУДИЈЕ

ПРЕДМЕТ: ИЗВЕШТАЈ КОМИСИЈЕ ЗА ОЦЕНУ И ОДБРАНУ ЗАВРШЕНЕ
ДОКТОРСКЕ ДИСЕРТАЦИЈЕ

Одлуком Наставно-научног већа Универзитета у Београду – Фармацеутског факултета, број 01.бр.1458/2, донетој на седници одржаној 18.6.2026. године, именована је Комисија за оцену и одбрану завршене докторске дисертације кандидата Милана Ј. Јовановића, магистра фармације, под називом „Хеминформатичка анализа, дизајн и испитивање инхибитора липидних и протеин киназа као потенцијално нових антинеопластика“, у следећем саставу:

1. Др сц. Бранка Ивковић, ванредни професор, Универзитет у Београду – Фармацеутски факултет (ужа научна област: Фармацеутска медицинска хемија) (Председник Комисије)
2. Др сц. Мара Алексић, редовни професор, Универзитет у Београду – Фармацеутски факултет (ужа научна област: Физичка хемија)
3. Др сц. Славица Ољачић, ванредни професор, Универзитет у Београду – Фармацеутски факултет (ужа научна област: Фармацеутска медицинска хемија)
4. Др сц. Марија Поповић Николић, виши научни сарадник, Универзитет у Београду – Фармацеутски факултет (ужа научна област: Фармација - Фармацеутска хемија)
5. Др сц. Бранислав Станковић, виши научни сарадник, Универзитет у Београду – Институт за нуклеарне науке „Винча“ (ужа научна област: Физичка хемија)

Ментори докторске дисертације су др сц. Катарина Николић, редовни професор, Универзитет у Београду – Фармацеутски факултет (ужа научна област: Фармацеутска медицинска хемија) и др сц. Милкица Цревар, ванредни професор, Универзитет у Београду – Фармацеутски факултет (ужа научна област: Фармацеутска медицинска хемија)

Комисија у наведеном саставу прегледала је докторску дисертацију и сачинила Извештај у складу са чланом 23. Правилника о докторским академским студијама Универзитета у Београду – Фармацеутског факултета који подноси Наставно-научном већу.

1. ОСНОВНИ ПОДАЦИ О КАНДИДАТУ И ДОКТОРСКОЈ ДИСЕРТАЦИЈИ

Кандидат: Милан Ј. Јовановић

Тренутно стручно/академско звање кандидата: магистар фармације

Установа: Универзитет у Београду – Фармацеутски факултет

Наслов докторске дисертације: „Хеминформатичка анализа, дизајн и испитивање инхибитора липидних и протеин киназа као потенцијално нових антинеопластика”

Научна област: Фармација

Ужа научна област: Фармацеутска медицинска хемија

Достављена електронска верзија дисертације садржи 223 стране. Основни текст дисертације организован је у седам поглавља: 1. Увод, 2. Циљ истраживања, 3. Експериментални део, 4. Резултати и дискусија, 5. Закључак, 6. Литература и 7. Прилози. Према нумерацији у тексту, дисертација садржи 48 слика/графичких приказа, 24 табеле и 520 библиографских јединица.

На почетку дисертације налазе се насловне стране на српском и енглеском језику, подаци о менторима и члановима Комисије, захвалница, сажетак на српском и енглеском језику, листа скраћеница и садржај, док се у завршном делу налазе прилози, биографија аутора и изјаве кандидата о ауторству, истоветности штампане и електронске верзије и коришћењу докторске дисертације.

2. ПРЕДМЕТ И ЦИЉЕВИ ДОКТОРСКЕ ДИСЕРТАЦИЈЕ

Предмет докторске дисертације је интегрисано експериментално и хеминформатичко испитивање одабраних инхибитора липидних и протеин киназа, као и структурно сродних једињења, са циљем бољег разумевања њихових молекулских особина, односа структуре и активности, као и различитих фармакокинетичких и токсиколошких особина. Истраживање обухвата процену пасивне пермеабилности инхибитора киназа кроз модел крвно-мождане баријере, електрохемијску карактеризацију ерлотиниба и његовог потенцијала за интеракцију са ДНК, као и рационални дизајн нових потенцијално селективних инхибитора *PI3K α* изоформе (енгл. *phosphatidylinositol 3-kinase alpha isoform*) применом савремених *CADD* (енгл. *Computer-Aided Drug Design*) метода.

Основни циљ докторске дисертације био је да се кроз повезивање експерименталних техника и рачунарских метода дефинишу структурни фактори који утичу на пермеабилност, редокс понашање, потенцијал интеракције са ДНК, активност и селективност киназних инхибитора. На тај начин дисертација обједињује анализу већ познатих представника циљане антитуморске терапије и дизајн нових једињења усмерених ка *PI3K α* као релевантном циљном месту дејства у развоју нових антинеопластика.

Специфични циљеви истраживања обухватали су:

- испитивање пасивне пермеабилности одабраних киназних инхибитора и експерименталних структурно сродних једињења кроз *in vitro* модел крвно-мождане баријере применом *PAMPA* (енг. *Parallel Artificial Membrane Permeability Assay*) теста;
- формирање, поређење, валидацију и интерпретацију *QSPR* (енгл. *Quantitative Structure-Property Relationship*) модела за предвиђање пермеабилности, уз примену *MLR* (енгл. *Multiple Linear Regression*), *ANN* (енгл. *Artificial Neural Network*) и *SVR* (енгл. *Support Vector Regression*) приступа;
- електрохемијску карактеризацију ерлотиниба на електроди од стакластог угљеника применом *CV* (енгл. *Cyclic Voltammetry*), *DPV* (енгл. *Differential Pulse Voltammetry*) и *SWV* (енгл. *Square-Wave Voltammetry*) техника;
- испитивање интеракције ерлотиниба са ДНК применом електрохемијског ДНК-биосензора, као и упоредну *in silico* анализу начина везивања ерлотиниба са ДНК применом молекулског докинга и молекулске динамике;
- развој и примену протокола за моделовање *PI3K* инхибитора селективних за α изоформу - молекулску динамику, ансамбл докинг и *3D-QSAR* (енгл. *Three-Dimensional Quantitative Structure-Activity Relationship*) анализу за *PI3K α* , *PI3K β* , *PI3K γ* и *PI3K δ* изоформи;
- дефинисање структурних смерница за дизајн нових потенцијално селективних *PI3K α* инхибитора, *in silico* процену њихове активности и селективности за *PI3K α* , као и додатну *in silico* процену њихових физичко-хемијских, фармакокинетичких и токсиколошких особина.

3. ПРИКАЗ САДРЖАЈА ДОКТОРСKE ДИСЕРТАЦИЈЕ

Структура докторске дисертације омогућава праћење истраживачког тока од теоријске основе о улози липидних и протеин киназа у малигним болестима, преко експерименталног и рачунарског испитивања одабраних киназних инхибитора, до рационалног дизајна селективних *PI3K α* инхибитора.

Поглавље „Увод” даје свеобухватан преглед малигнух тумора, структуре и класификације протеин киназа, улоге липидних киназа и *PI3K* сигналног пута, као и значаја *PI3K* инхибитора у терапији малигнух тумора. Посебна пажња посвећена је *PI3K α* селективним инхибиторима, изазовима постизања изоформне селективности и значају оптимизације фармакокинетичког и токсиколошког профила. У наставку увода приказане су методе рачунарског дизајна лекова засноване на структури циљног места и структури лиганда, укључујући хомологно моделовање, молекулски докинг, молекулску динамику, виртуелни скрининг, *QSAR/QSPR* анализе, машинско учење и дефинисање домена применљивости развијених модела. У завршном делу увода обрађени су терапијски потенцијал и изазови проласка киназних инхибитора кроз крвно-мождану баријеру, као и могућност интеракције киназних инхибитора са ДНК и њихово електрохемијско испитивање.

Поглавље „Циљ истраживања” дефинише основни циљ рада и специфичне задатке који су распоређени у три истраживачке целине: *PAMPA*-КМБ/*QSPR* анализу пермеабилности, електрохемијско и *in silico* испитивање ерлотиниба и његове интеракције са ДНК, као и *CADD* студију усмерену на дизајн и селекцију потенцијално селективних *PI3K α* инхибитора.

Поглавље „Експериментални део” обухвата опис коришћених стандарда, хемикалија, опреме и рачунарских програма, као и методолошке протоколе за све експерименталне и рачунарске поступке. У првом делу описани су *PAMPA*-КМБ тест, *HPLC* (енгл. *High-Performance Liquid Chromatography*) анализа резултата, припрема скупа података, израчунавање молекулских дескриптора, развој *MLR*, *ANN* и *SVR QSPR* модела, њихова валидација и дефинисање домена применљивости. У другом делу приказани су поступци волтаметријских испитивања, припрема ДНК-биосензора, као и протоколи молекулског докинга и молекулске динамике за испитивање интеракције ерлотиниба са ДНК. У трећем делу описани су поступци припреме сетова података, хомологног моделовања, молекулске динамике, ансамбл докинга, *3D-QSAR* моделовања заснованог на *PLS* (енгл. *Partial Least Squares*) методологији, *LDA-SBVS* (енгл. *Linear Discriminant Analysis-Structure-Based Virtual Screening*) виртуелног скрининга, процене *ADMET* (енгл. *Absorption, Distribution, Metabolism, Excretion and Toxicity*) особина и додатне примене *PAMPA-SVR* модела на новодизајнирана и једињења добијена виртуелним скринингом, као и структурне анализе са циљем процене могућег редокс понашања и примене волтаметријских испитивања дизајнираних једињења.

Поглавље „Резултати и дискусија” подељено је на три тематски повезане целине. Прва целина обухвата резултате *PAMPA*-КМБ *QSPR* студије, укључујући експерименталне вредности пермеабилности кроз КМБ, формирање и интерпретацију

QSPR модела и поређење са доступним литературним и фармакокинетичким подацима. Друга целина приказује резултате електрохемијске студије ерлотиниба, анализу оксидоредукционих процеса, испитивање интеракције са ДНК и рачунарску процену могућих начина везивања. Трећа целина посвећена је *PI3K* дизајн студији, од хомологног моделовања *PI3K*β изоформе, молекулске динамике и валидације докинг протокола, преко развоја *3D-QSAR* модела за *PI3K*α, *PI3K*β, *PI3K*γ и *PI3K*δ изоформе, до дефинисања структурних смерница, дизајна нових једињења применом *scaffold hopping* методе, виртуелног скрининга, анализе хемијског простора и процене *ADMET* особина, укључујући примену *PAMPA*-КМБ *QSPR* модела, као и структурне анализе уз процену могућег редокс понашања и потенцијалну корелацију са волтаметријским испитивањем дизајнираних једињења. У оквиру резултата су предложени најбољи кандидати за даља истраживања.

Поглавље „Закључак” систематично сумира најважније резултате и научне доприносе дисертације. Закључци су формулисани у складу са постављеним циљевима и јасно повезују експерименталне налазе, валидацију добијених резултата и структурне смернице за даљи развој киназних инхибитора.

Поглавље „Литература” садржи 520 библиографских јединица, што указује на темељно обрађену релевантну литературу из области фармацеутске-медицинске хемије, *CADD* метода, *QSAR/QSPR* моделовања, *PI3K* инхибитора, испитивања пермеабилности кроз КМБ и електрохемијских метода у фармацеутској анализи.

Поглавље „Прилози” садржи додатне податке које употпуњују резултате из дисертације, укључујући структуре испитиваних једињења, као и њихове експерименталне и предвиђене активности

4. ПРЕГЛЕД РЕЗУЛТАТА ПРЕДСТАВЉЕНИХ У ДОКТОРСКОЈ ДИСЕРТАЦИЈИ

У првој истраживачкој целини испитана је ефективна пермеабилност 34 структурно различита једињења применом *PAMPA*-КМБ теста. Скуп података обухватио је 15 одобрених инхибитора протеин киназа, N-дезметилиматиниб (иматиниб *EP* нечистоћу *C*), експериментални *ROCK* (енгл. *Rho-Associated Coiled-Coil Containing Protein Kinase*) инхибитор *RKI-1447*, као и структурно сродна експериментална једињења која укључују деривате арил-2-аминопиридина, тиазолил-хидразона и селеназолил-хидразона. Овакав избор једињења омогућио је да се испитају различити структурни делови присутни у киназним инхибиторима и сродним једињењима.

На основу експериментално одређених $\log P_e$ вредности, испитивана једињења су класификована у групе ниске, средње и високе пермеабилности. Добијени резултати су показали да се киназни инхибитори међусобно значајно разликују у способности пасивне дифузије кроз мембрански модел КМБ, што је у складу са структурном разноврсношћу ове групе лекова. Поређење *PAMPA* резултата са доступним клиничким и фармакокинетичким подацима указало је да пасивна мембранска пермеабилност представља важан, али не и једини фактор који одређује пролазак лекова у ЦНС, јер на дистрибуцију додатно утичу активни транспорт, везивање за протеине плазме, и метаболизам.

За опис и предвиђање пермеабилности развијени су *QSPR* модели применом *MLR*, *ANN* и *SVR* приступа. *MLR* модел је издвојио четири кључна дескриптора: *CATS2D_09_DA*, *CATS2D_04_AA*, *B04[N-S]* и *F07[C-N]*. Дескриптор *CATS2D_04_AA* имао је најизраженији позитиван допринос $\log P_e$ вредности, док су *B04[N-S]* и *F07[C-N]* такође позитивно корелирали са пасивном пермеабилношћу. Насупрот томе, *CATS2D_09_DA* имао је негативан утицај, што указује да одређени тополошки распоред донорских и акцепторских центара може неповољно утицати на пролаз кроз модел КМБ.

Након поређења статистичких параметара, најпоузданијим моделом оцењен је *QSPR-SVR* модел, са вредностима $R^2 = 0,839$, $Q^2 = 0,648$ и $R^2_{\text{pred}} = 0,777$. Овај модел је задовољио кључне критеријуме интерне и екстерне валидације, а графици одзива указали су на постојање нелинеарних односа између појединих дескриптора и $\log P_e$ вредности. Дефинисање домена применљивости омогућило је процену поузданости предикција и додатно подржало примену модела као алата за прелиминарни избор нових киназних инхибитора са повољним профилем пермеабилности кроз КМБ.

У другој истраживачкој целини ерлотиниб је испитан као представник киназних инхибитора 4-анилинохиназолинског типа, ради процене његовог редокс понашања и потенцијала за интеракцију са ДНК. Електрохемијска карактеризација спроведена је на немодификованој електроди од стакластог угљеника применом цикличне волтаметрије, диференцијално пулсне волтаметрије и волтаметрије правоугаоних таласа. Испитиван је утицај рН вредности, концентрације ерлотиниба и брзине промене потенцијала на положај и интензитет оксидационих и редукционих пикова.

Резултати су показали да су редокс процеси ерлотиниба претежно иреверзибилни. Најизраженији оксидациони процес повезан је са секундарном ароматичном аминок групом, која је на основу експерименталних резултата издвојена као највероватније електроактивно место. Анализа утицаја брзине промене потенцијала указала је да је главни анодни процес претежно контролисан адсорпцијом, док је за поједине редукционе процесе уочен мешовит дифузионо-адсорпциони карактер. Испитивање утицаја рН допринело је разумевању броја протона и електрона укључених у редокс процесе и омогућило претпоставку о могућем механизму оксидационих и редукционих реакција.

Интеракција ерлотиниба са ДНК испитана је применом електрохемијског ДНК-биосензора. Пад струје деоксиаденозинског сигнала у присуству растућих концентрација ерлотиниба указао је на интеракцију овог једињења са ДНК. Израчуната константа везивања износила је $K = 8,25 \times 10^4 \text{ M}^{-1}$, док је мали померај потенцијала пика указао на могућност делимично интеркалационог начина везивања и/или везивања у жлеб ДНК. Ови резултати су допуњени молекулским докинг и молекулском динамиком, који су показали да ерлотиниб може да се веже и интеркалацијом и у мали жлеб ДНК, али да су рачунарски резултати указали на интеркалацију као вероватнији начин стабилног везивања.

Ова целина дисертације има методолошки значај јер повезује класичне електроаналитичке технике, ДНК-биосензор и рачунарске методе у процени потенцијалних интеракција киназних инхибитора са ДНК. Добијени резултати представљају основу за будућа испитивања структурно сродних инхибитора киназа, укључујући дизајниране *PI3K* инхибиторе који садрже хетероцикличне фрагменте и потенцијално електроактивне функционалне групе.

Трећа и најобимнија истраживачка целина посвећена је рационалном дизајну нових потенцијално селективних инхибитора *PI3K*α изоформе. Основни сет испитиваних инхибитора укључивао је једињења из бензоксазепинског и тиазолног кластера, која су коришћена за анализу односа структуре, активности и изоформне селективности. Будући да је процена селективности захтевала испитивање могућег *off-target* везивања *PI3K*α-усмерених инхибитора за *PI3K*β, *PI3K*γ и *PI3K*δ изоформе, у дисертацији су анализиране све четири *PI3K* изоформе класе I. За *PI3K*β изоформу је, услед недоступности одговарајуће хумане кристалографске структуре, израђен хомологни модел на основу кристалне структуре 2Y3A. Валидација хомологног модела и каснија анализа молекулске динамике потврдиле су његову применљивост у даљим докинг и QSAR студијама.

Молекулски докинг је валидиран докинг лиганада из кристалографских структура, при чему су добијене *RMSD* вредности биле ниже од 2 Å за све испитиване *PI3K* изоформе. Након тога су припремљени комплекси референтних лиганада са *PI3K*α, *PI3K*β, *PI3K*γ и *PI3K*δ изоформама, а молекулска динамика је коришћена за избор репрезентативних конформација активног места. Кластери добијени из трајекторија молекулске динамике послужили су за ансамбл докинг, што је омогућило да се у анализу укључи флексибилност активног места и да се добију реалистичније везивне позе лиганада.

На основу одабраних везаних конформација развијени су *3D-QSAR* модели за све четири *PI3K* изоформе. Модели су показали високе вредности параметара интерне и екстерне валидације, са вредностима R^2 у опсегу 0,93–0,96, Q^2 у опсегу 0,77–0,88 и R^2_{pred} у опсегу 0,71–0,75. Додатно су примењене методе валидације као што су *r*-метрика, *CCC* и анализа домена применљивости, што је потврдило статистичку стабилност и поузданост формираних модела. Интерпретација најважнијих *GRIND-2* променљивих формираних модела омогућила је дефинисање кључних структурних карактеристика испитиваних једињења које утичу на активност и селективност на *PI3K α* , као и дизајн селективних *PI3K α* инхибитора.

За *PI3K α* *3D-QSAR* модел идентификовани су повољни доприноси везани за просторни распоред хидрофобних, стерних и водонично-везујућих карактеристика, што је повезано са правилним позиционирањем фрагмената лиганда у зглобном региону, афинитетном цепу и региону у ком се везује модификатор селективности. Посебно значајне интеракције обухватају водоничне везе са *Val851*, *Ser854*, *Thr856* и *Gln859*, као и хидрофобне контакте са остацима *Met772*, *Ile800*, *Tyr836*, *Ile848*, *Val850*, *Met922*, *Phe930* и *Ile932*. Упоредна анализа *PI3K β* , *PI3K γ* и *PI3K δ* модела омогућила је идентификацију карактеристика које треба избегавати ради смањења *off-target* активности и побољшања селективности ка *PI3K α* .

На основу интегрисане анализе *3D-QSAR* модела и молекулског докинга дефинисане су смернице за дизајн нових *PI3K α* инхибитора. У оквиру стратегије *scaffold hopping* и структурних модификација *R*-остатака изабрани су нови структурни фрагменти за везивање у зглобном региону, афинитетном цепу и региону у ком се везује модификатор селективности за *PI3K α* . Дизајнирана су 23 нова једињења, а њихова активност и селективност за *PI3K α* процењене су ансамбл докингом и *3D-QSAR* анализом. Поред тога, спроведен је *LDA-SBVS* виртуелни скрининг *Enamine* библиотеке киназних инхибитора, након чега је осам једињења издвојено као потенцијално активни и селективни *PI3K α* инхибитори.

Додатно су анализирани хемијски простор, физичко-хемијске особине, сложеност синтезе, *ADMET* профил и предвиђена пермеабилност кроз КМБ. *PCA* (енгл. *Principal Component Analysis*) анализа показала је да дизајнирана једињења покривају најшири и најразноврснији хемијски простор у односу на полазне кластере *PI3K* инхибитора, док су *VS* једињења заузимала делимично издвојен регион. Коначна интегрисана процена, укључујући анализу *ADMET* карактеристика процена довела је до издвајања **D4**, **D5**, **D23** и **VS1** као најперспективнијих *PI3K α* инхибитора и кандидата за даљу оптимизацију.

Применом претходно развијеног *PAMPA-SVR QSPR* модела из прве целине дисертације додатно је процењена пасивна пермеабилност новодизајнираних и *VS* једињења. Водеће једињење **B1** сврстано је у групу једињења средње пермеабилности, док је више дизајнираних једињења показало једнаку или већу предвиђену пермеабилност. Сва испитивана једињења налазила су се у домену применљивости *PAMPA-SVR QSPR* модела, што подржава поузданост добијених предикција и показује вредност интеграције *QSPR* модела пермеабилности са дизајном нових *PI3K α* инхибитора.

5. УПОРЕДНА АНАЛИЗА РЕЗУЛТАТА КАНДИДАТА СА ПОДАЦИМА ИЗ ЛИТЕРАТУРЕ

Резултати докторске дисертације су у значајној мери усклађени са актуелним правцима медицинске хемије киназних инхибитора, у којима се примењују *in silico* методе, анализа односа структуре и активности, молекулско моделовање и рана процена фармакокинетичких особина [1].

У делу који се односи на пермеабилност кроз КМБ, дисертација се надовезује на савремене приступе раног профилисања ЦНС пермеабилности киназних инхибитора, што је у складу са радом *Di* и сар. [2], у коме је *PAMPA*-КМБ приступ представљен као брз модел за процену пасивне дифузије кроз КМБ. Добијени *PAMPA*-КМБ и *QSPR* резултати могу се тумачити у оквиру општих принципа који одређују пролазак једињења кроз КМБ. *Zhang* и сар. [3], *Barn* и сар. [4] и *Wager* и сар. [5] показали су да ЦНС пермеабилност зависи од више међусобно повезаних особина, укључујући липофилност, поларност, молекулску величину, способност формирања водоничних веза, јонизационе карактеристике и тополошке дескрипторе. У односу на наведене опште моделе ЦНС пермеабилности, *PAMPA*-КМБ *QSPR* модели развијени у дисертацији засновани су на скупу структурно разноврсних киназних инхибитора и њима сродних једињења, због чега пружају додатни увид у тополошке и структурне карактеристике значајне за ову групу једињења [6]. Ово је посебно важно јер поларни хетероциклични фрагменти киназних инхибитора често учествују у везивању за активно место ензима, па оптимизација пермеабилности мора бити разматрана заједно са очувањем афинитета и селективности према циљаној кинази.

Део докторске дисертације посвећен електрохемијским испитивањима заснива се на значају електрохемијских метода у испитивању редокс понашања лекова и њихових интеракција са ДНК. *Chiorcea-Paquim* и *Oliveira-Brett* [7], као и *Ramotowska* и сар. [8], приказали су да електрохемијски приступи, укључујући ДНК-биосензоре, могу бити корисни за испитивање интеракција малих молекула са ДНК. Овакав приступ је релевантан и за киназне инхибиторе, јер поједини представници ове групе садрже хетероароматске системе и функционалне групе које могу учествовати у редокс процесима или интеракцијама са ДНК. У том контексту, претходно су испитиване интеракције лапатиниба [9], дасатиниба [10] и сунитиниба [11] са ДНК. Код ерлотиниба, *Bakırhan* и сар. [12] су испитивали његово оксидационо понашање, волтаметријско одређивање и извршили *DFT* (енгл. *Density Functional Theory*) анализу као подршку предложеном механизму оксидације, док су *Chen* и сар. [13] испитивали интеракцију ерлотиниба са ДНК применом спектроскопских метода. У односу на наведене радове, у дисертацији је анализа ерлотиниба проширена повезивањем волтаметријских резултата, електрохемијског ДНК-биосензора, молекулског докинга и молекулске динамике. На тај начин су редокс понашање ерлотиниба, његов потенцијал за интеракцију са ДНК и молекулска основа добијених експерименталних резултата сагледани у оквиру јединственог експериментално-рачунашког приступа [14].

Протокол који је примењен за дизајн селективних *PI3K* инхибитора у складу је са доступним литературним подацима у којим се истиче значај изоформно селективних *PI3K α* инхибитора који показују антитуморско дејство уз повољнији безбедносни профил. Значај овог приступа потврђују примена клинички одобрених *PI3K α* инхибитора, као што су алпелисиб [15] и инаволисиб [16], као и других *PI3K* инхибитора који се налазе у различитим фазама клиничких студија. У поређењу са алпелисибом, чија је селективност у литератури описана преко *IC*₅₀ вредности, више дизајнираних једињења је показало повољан предвиђени профил селективности ка *PI3K α* у односу на *PI3K β* , *PI3K γ* и *PI3K δ* [17]. У поређењу са инаволисибом, за који су у литератури доступне *pK_i* вредности и подаци о израженој селективности ка *PI3K α* , код дизајнираних једињења предвиђена селективност је била мање наглашена, али је и даље указивала на јасну усмереност ка *PI3K α* изоформи [18]. Овакво поређење са клинички релевантним *PI3K α* инхибиторима показује да је примењена стратегија дизајна довела до структурно нових једињења са релевантним селективним профилем, чиме се додатно потврђује иновативност дисертације.

Структурна основа селективности тумачена је у складу са радовима *Frazzetto* и сар. [19] и *Zheng* и сар. [20], који су указали на значај неконзервисаних аминокиселинских остатака у каталитичком цепу *PI3K* изоформи. Значај укључивања флексибилности активног места додатно је подржан резултатима добијеним криоелектронском микроскопијом, који показују да *PI3K α* може зауимати више конформационих стања [21]. Новије *in silico* студије *PI3K α* инхибитора комбинују различите методологије са фокусом на моделовање *PI3K α* инхибиције. *Halder* и сар. [22] су применили фрагментно заснован дизајн, биоизостерну замену, докинг, *MM/GBSA* (енгл. *Molecular Mechanics/Generalized Born Surface Area*), *DFT*, молекулску динамику и *ADMET* анализу. *Dibia* и сар. [23] су комбиновали молекулски докинг и *3D-QSAR* моделовање, док су *Wang* и сар. [24] користили *3D-QSAR*, *ADMET* предвиђања, докинг и молекулску динамику. Ова дисертација примењује свеобухватнији *CADD* протокол који обухвата моделовање све четири изоформе - *PI3K α* , *PI3K β* , *PI3K γ* и *PI3K δ* . Исти протокол укључује и хомологно моделовање *PI3K β* , молекулску динамику, ансамбл докинг, *3D-QSAR* моделовање *PI3K α* , *PI3K β* , *PI3K γ* и *PI3K δ* изоформе, као и виртуелни скрининг и *ADMET* профилисање. На основу тога су дефинисане структурне смернице за очување активности ка *PI3K α* уз смањење вероватноће неповољног везивања за *PI3K β* , *PI3K γ* и *PI3K δ* [25].

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6. ОБЈАВЉЕНИ И САОПШТЕНИ РЕЗУЛТАТИ КОЈИ ЧИНЕ ДЕО ДОКТОРСКЕ ДИСЕРТАЦИЈЕ

Рад у водећем међународном часопису (M21)

Jovanović, M.; Nikolić, K.; Čarapić, M.; Aleksić, M.M. Electrochemical and Theoretical Study on Interaction between Erlotinib and DNA. *J. Pharm. Biomed. Anal.* 2023, 234, 115560, doi:10.1016/j.jpba.2023.115560.

(M21, *Chemistry, Analytical* (24/87), IF2021 = 3,571)

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(M21, *Chemistry, Medicinal* (16/63), IF2022 = 4,2)

Рад у националном часопису (M52)

Jovanović, M.; Nikolić, K.; Gagić, Ž.; Agbaba, D. Molecular Modeling and Analysis of the 3D Pharmacophore Structure of the Selective PI3K- α Inhibitors as Antitumor Agents. *Arh. Farm.* 2018, 68, 860–873, doi:10.5937/ArhFarm1804860J.

Саопштења са међународних скупова штампана у целини (M33)

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7. ОБРАЗЛОЖЕЊЕ НАУЧНОГ ДОПРИНОСА ДОКТОРСКЕ ДИСЕРТАЦИЈЕ

Докторска дисертација кандидата магистра фармације Милана Ј. Јовановића представља оригинално и интердисциплинарно истраживање у области фармацеутске медицинске хемије, усмерено на хеминформатичку анализу, експериментално испитивање и рационални дизајн инхибитора липидних и протеин киназа као потенцијално нових антинеопластика. Рад обједињује експерименталне методе, електроаналитичке технике, ДНК-биосензоре, молекулски докинг, молекулску динамику, *3D-QSAR/QSPR* моделовање, машинско учење, виртуелни скрининг и *ADMET* профилисање, што дисертацији даје јасно мултидисциплинарно обележје.

Научни допринос прве целине огледа се у експерименталном испитивању пасивне пермеабилности великог и структурно разноврсног скупа киназних инхибитора и сродних једињења, као и у развоју валидираног *PAMPA-SVR* модела који се може користити за рану процену пермеабилности нових киназних инхибитора кроз модел КМБ. Анализа дескриптора омогућила је разумевање утицаја тополошког распореда донорских и акцепторских центара, као и специфичних атомских парова, на пасивну пермеабилност.

Научни допринос друге целине огледа се у детаљној електрохемијској карактеризацији ерлотиниба, дефинисању највероватнијег електроактивног места главног оксидационог процеса и процени његове интеракције са ДНК. Комбиновање волтаметријских техника, ДНК-биосензора и рачунарских метода омогућило је да се експериментални резултати тумаче у ширем структурном и механистичком контексту.

Научни допринос треће целине односи се на развој интегрисаног *CADD* протокола за дизајн нових потенцијално селективних *PI3K α* инхибитора. У дисертацији су развијени и валидирани *3D-QSAR* модели за *PI3K α* , *PI3K β* , *PI3K γ* и *PI3K δ* изоформе, дефинисане су структурне детерминанте активности и селективности, дизајнирана су 23 нова једињења, а виртуелним скринингом издвојено је осам потенцијално активних *PI3K α* инхибитора. Интегрисаном проценом предвиђене активности, селективности, *ADMET* особина и пермеабилности издвојена су једињења **D4**, **D5**, **D23** и **VS1** као најперспективнији кандидати за даљу оптимизацију.

Укупно посматрано, дисертација доприноси разумевању односа структуре и дејства киназних инхибитора, као и развоју нових приступа за рачунарски дизајн *PI3K α* инхибитора као циљаних антинеопластика. Добијени резултати имају значај за даља истраживања у области циљане антитуморске терапије, посебно у контексту избора једињења са повољним профилем активности, селективности, пермеабилности и *ADMET* карактеристика.

8. ПРОВЕРА ОРИГИНАЛНОСТИ ДОКТОРСКЕ ДИСЕРТАЦИЈЕ

ИЗВЕШТАЈ О ПРОВЕРИ ОРИГИНАЛНОСТИ ДОКТОРСКЕ ДИСЕРТАЦИЈЕ

У складу са *Правилником о поступку провере оригиналности докторских дисертација које се бране на Универзитету у Београду*, и на основу резултата анализе добијених применом софтверског програма *iThenticate*, извршена је провера оригиналности докторске дисертације под називом: **„Хеминформатичка анализа, дизајн и испитивање инхибитора липидних и протеин киназа као потенцијално нових антинеопластика”**, коју је поднео кандидат магистар фармације Милан Јовановић.

Утврђени степен текстуалног подударања износи 5%. Највећа подударност од 1% утврђена је са претходно публикованим радом кандидата који је саставни део докторске дисертације. Остале подударности су последица понављања цитата, имена аутора, библиографских навода коришћене литературе, опште познатих формулација и научних података, као и претходно објављених резултата самог кандидата који су саставни део дисертације. Сви наведени елементи у потпуности су у складу са чланом 9. наведеног Правилника.

На основу свега изнетог, а у складу са чланом 8. став 2. Правилника о поступку провере оригиналности докторских дисертација које се бране на Универзитету у Београду, изјављујемо да извештај указује на оригиналност докторске дисертације, те се прописани поступак припреме за њену одбрану може наставити.

Датум

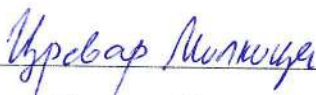
30.06.2026. године

Ментори:



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9. ИЗЈАВА МЕНТОРА И КАНДИДАТА ДА ИСТРАЖИВАЊЕ ЗА ПОТРЕБЕ ДОКТОРСКЕ ДИСЕРТАЦИЈЕ НИЈЕ РАЂЕНО У ЗДРАВСТВЕНОЈ УСТАНОВИ

Изјављујемо да истраживање за потребе израде докторске дисертације под насловом:

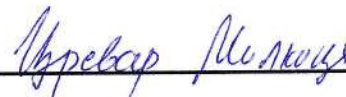
„Хеминформатичка анализа, дизајн и испитивање инхибитора липидних и протеин киназа као потенцијално нових антинеопластика”

није рађено у здравственој установи.

Потписи ментора:



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Потпис кандидата:



Маг.фарм. Милан Јовановић

У Београду, дана 30.6.2026.године

10. ПРЕДЛОГ КОМИСИЈЕ ЗА ОЦЕНУ ЗАВРШЕНЕ ДОКТОРСKE ДИСЕРТАЦИЈЕ

На основу прегледа докторске дисертације, Комисија закључује да је кандидат магистар фармације Милан Ј. Јовановић успешно реализовао постављене циљеве истраживања, применио савремене експерименталне и рачунарске методе и остварио оригиналан научни допринос у области фармацеутске медицинске хемије. Дисертација је написана јасно и прегледно, резултати су систематично приказани и критички дискутовани, а закључци су у складу са добијеним резултатима.

Комисија сматра да докторска дисертација под називом „Хеминформатичка анализа, дизајн и испитивање инхибитора липидних и протеин киназа као потенцијално нових антинеопластика” испуњава услове прописане Законом о високом образовању, Статутом Универзитета у Београду, Статутом Универзитета у Београду – Фармацеутског факултета и Правилником о докторским академским студијама Универзитета у Београду – Фармацеутског факултета.

Комисија предлаже Наставно-научном већу Универзитета у Београду – Фармацеутског факултета да прихвати позитиван Извештај о оцени завршене докторске дисертације и одобри кандидату Милану Ј. Јовановићу јавну одбрану докторске дисертације.

У Београду, 2.7.2026.



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Electrochemical and theoretical study on interaction between erlotinib and DNA

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ABSTRACT

A comprehensive investigation of tyrosine kinase inhibitor erlotinib (ERL) electrochemical behavior and interaction with DNA was performed with the aim to clarify its redox mechanism and to determine the mode of binding. Irreversible oxidation and reduction processes of ERL on glassy carbon electrode were investigated using three voltammetric techniques CV, DPV, SWV in pH range between 2.0 and 9.0. Oxidation was established as an adsorption-controlled process, while the reduction manifested diffusion-adsorption mixed controlled process in acidic medium and adsorption became predominant in the neutral solutions. According to the determined number of transferred electrons and protons, oxidation and reduction mechanism of ERL are proposed. To follow the interaction between ERL and DNA, the multilayer ct-DNA electrochemical biosensor was incubated in ERL solutions concentrations ranged from 2×10^{-7} M to 5×10^{-5} M (pH 4.6) for 30 min. SWV measurements have shown the decrease in deoxyadenosine peak current as a consequence of ERL increased concentration and binding to ct-DNA. The calculated value of binding constant was $K = 8.25 \times 10^4 \text{ M}^{-1}$. Molecular docking showed that ERL forms hydrophobic interactions when docked into minor groove, as well as when intercalated, and molecular dynamics analysis predicted the stability of obtained complexes. These results together with voltammetric studies imply that the intercalation could be more dominant way ERL binding to DNA compared to minor groove binding.

1. Introduction

Erlotinib is a tyrosine kinase inhibitor used as first line therapy of non-small cell lung cancer and in combination with gemcitabine at the pancreatic cancer [1]. It belongs to the class of reversible EGFR/HER1 (human epidermal growth factor receptor type I) kinase inhibitors type I and binds to the active form of enzyme. Erlotinib contains 4-anilinequinazoline pharmacophore with oxygen containing substituents at C-6 and C-7 (Fig. 1).

The high selectivity for EGFR kinase is provided with electron withdrawing substituent at position 3' of the aniline phenyl ring and 4' unsubstituted position and it binds to hydrophobic pocket of the active form of the enzyme [2]. It inhibits intracellular phosphorylation of

tyrosine kinase through an ATP (adenosine triphosphate) - competitive binding to the kinase domain. Expression increases of epidermal growth factor and its receptor (EGFR) are key targets in such tumors and its activation is linked with increased proliferation, decreased apoptosis, angiogenesis, and metastasis [3]. EGFR mutations have an important role in the pathogenesis of non-small cell lung cancer. Combined with gefitinib, erlotinib could be used in patients with uncommon EGFR mutations [4]. Very recently, some erlotinib off-label uses were reported in the literature which relate to the mechanism of action. The erlotinib was effective in kidney renal cell carcinoma and the role of erlotinib's therapeutic targets were investigated [5]. Erlotinib gold conjugate also showed high cytotoxicity in breast cancer cell lines [3]. Erlotinib-hydrochloride is used oral as tablet and it shows pH dependent

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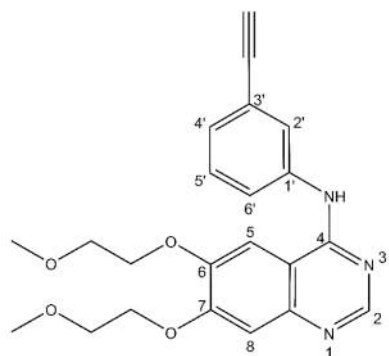
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N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine

Fig. 1. Structure and nomenclature of erlotinib.

solubility, higher at lower pH due to protonation of secondary amine. It shows extensive CYP3A4 metabolism (O-dealkylation) and further oxidation which leads to variable bioavailability [6]. The most common side effects are rash, diarrhea, loss of appetite, tiredness, and also some effects on eyes like serious cases of keratitis and ulcerative keratitis, on ears (bilateral eardrum perforation) and on liver (acute hepatitis) are reported [7].

There are several mechanisms of action for anticancer drugs targeting inhibition of protein kinase in order to achieve modification of DNA of cancer cells [8]. According to electrophilic theory genotoxic compounds could bind covalently to the DNA rich with electrons. This type of reaction is irreversible and leads to complete inhibition of DNA functions and subsequent cell death. Another, more desirable, type of binding which is reversible and leads only to change in the DNA conformation and morphology is non-covalent binding of drugs to DNA. Several modes of non-covalent interaction may occur between drug and DNA molecule, such as intercalation, binding to minor and major groove, or electrostatic binding to negative sugar-phosphate DNA backbone [9]. The stability of the formed DNA-drug complex depends on the intensity of the mentioned interactions.

Plenty of techniques are used to investigate mode and intensity of DNA-drug interactions from classical UV–VIS spectroscopy, along with fluorescence, IR and NMR spectroscopy, all the way to most recent optical and electrochemical biosensors. Although spectroscopic methods are traditionally used for studying drug–DNA interactions, they have some disadvantages. Those methods, especially with biological samples, often need long lasting and demanding pretreatment and the results may be affected by the turbidity of a sample. On the other hand, electrochemical techniques are performed with higher speed and reliability, and require small number of samples. In addition to all this, one of the main advantages of electrochemical methods is in their ability to measure and determine the binding constants over a wider range of concentration.

Thus, investigation of electrochemical mechanisms of tyrosine kinase inhibitors are important for biological systems related to electron transport chain and to better understand the relationship between structure and mechanism of action [10]. Oxidation of ERL has recently been investigated by using a graphite rod electrode modified by the nitrogen doped graphene quantum dots (N-GQDs) and a ternary nanohybrid comprising copper nanoparticles, polyaniline, along with graphene oxide, and oxidation mechanism in the acidic medium has been proposed [11]. Unlike oxidation, there is no literature data on ERL reduction process. The investigation of the interaction of several tyrosine kinase inhibitors with DNA with different methodologies has been assessed. Lapatinib interaction with double-stranded DNA was investigated with various voltammetric techniques such as different pulse voltammetry (DPV), square wave voltammetry (SWV) and cyclic voltammetry (CV) and it was confirmed that it could intercalate into the dsDNA helix [12]. The interaction between ibrutinib and dsDNA was

investigated with the use of voltammetric and molecular docking techniques and the groove binding mode to the DNA was suggested [13]. The characterization of binding interaction between erlotinib and Calf Thymus DNA (ct-DNA) in vitro using multi-spectroscopic methodologies, viscosity measurement and molecular docking and density functional theory was investigated and suggest that the main is hydrophobic interaction [14]. A recent paper [15] presented biocompatible, ultra-sensitive electrochemical DNA biosensor based on poly-L-methionine and bimetallic AuPt nanoparticles to detect Imatinib and ERL in picomolar concentration. The interaction between ERL and ds-DNA onto the biosensor surface, characterized as electrostatic binding, was used for determination of ERL concentration within the range of 0.032–1.0 nM and applied for detecting in human serum and pharmaceutical samples.

The aim of this study was to examine and clarify possible oxidation and reduction mechanism of erlotinib on glassy carbon electrode using three voltammetric techniques CV, DPV, SWV, and to investigate its interaction with DNA. For this purpose, DNA electrochemical biosensor was used. It is a device that integrates DNA as the biological recognition element and an electrode as the physicochemical transducer, i.e., an electrode chemically modified by nucleic acid. Several preparation methods are available regarding to immobilization technique, to produce thin, thick or multi-layer biosensor [16]. Voltammetric techniques, especially the pulse ones (SWV and DPV), are highly sensitive and are useful for investigating interaction of in situ generated reactive species (by compound redox reactions on the electrode surface) with biological material immobilized on the electrode surface [17].

In this study, we used multi-layer ct-DNA electrochemical biosensor, and SWV technique for analysis of interactions between erlotinib and DNA. The results of this research will contribute to the understanding of the mechanism of interaction between erlotinib and nucleic acid. This study investigates the interaction between erlotinib and ct-DNA using voltammetric techniques at bare glassy carbon electrode and the determination of binding constant for the first time. Molecular docking and molecular dynamics analysis were used to verify and support the experimental findings.

2. Material and methods

2.1. Apparatus

The μ -Autolab analyser operating with GPES 4.9 software (Eco Chemie, Utrecht, The Netherlands) was used for performing voltammetric measurements. Glassy carbon electrode (GCE, $d = 3$ mm, CH Instruments, Inc., Austin, TX, USA) was used as a working electrode, together with Ag/AgCl as a reference (3.00 M KCl) and Pt as an auxiliary electrode inside three-electrode electrochemical cell. Prior to measurement GCE was prepared each time by polishing it on a smooth polishing pad with aqueous slurry of Al_2O_3 powder (particle size 0.05 μm). The GCE was sonicated after polishing in deionized water and absolute ethanol for 2 min in each. This was performed in an ultrasonic bath 'Iskra' UZ 4 R (Sentjernej, Slovenia) and pH measurements were performed using PHM 220 pH meter with combined electrode Radiometer GK2401B. Weight measurements were carried out with a SCALTEC SBC 32 balance.

2.2. Reagents and solutions

Deoxyribonucleic acid sodium salt from calf thymus (Calf thymus DNA (ct-DNA) Product Number D 1501) and erlotinib-hydrochloride were obtained from Sigma Aldrich. Sodium citrate dehydrate and citric acid produced by Fisher scientific, and sodium acetate, acetic acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, ammonia and ammonium chloride from Sigma Aldrich, were of analytical grade purity (p.a.) chemicals. The stock solution of DNA, 73.95 $\mu\text{g/mL}$, was prepared by dissolving it in 0.1 M acetate buffer, pH 4.6. The composition (content of fractions other than dsDNA), purity

and stability and of DNA sample have been examined and validated in our previous work [18], using agarose gel electrophoresis, UV–VIS spectrophotometry and fluorimetric Qubit BR dsDNA assay. A stock solution of 1×10^{-3} M ERL was prepared in methanol and stored at 4 °C. Final solutions of 2×10^{-4} M concentration of ERL were prepared *ex tempore* by diluting the stock solution in different buffers. Citrate (pH 2.20 and 3.06), acetate (pH 3.58, 4.64, 5.05 and 5.64), phosphate (pH 5.89, 6.94 and 8.05) and ammonia (pH 8.51 and 8.90) buffers with ionic strength of 0.10 M (prepared using deionized water) were used for the preparation. All the experiments were performed at room temperature (25 °C). High purity nitrogen gas was set to pass through solutions in the cell for 10 min before performing each measurement to eliminate oxygen from the solutions, as well as above the solution during the measurements.

2.3. Preparation of the biosensor

The multilayer ct-DNA biosensor was prepared by adding three 5 μ L drops of DNA stock solution onto previously prepared GCE, with each drop drying for 30 min under an atmosphere of nitrogen. After drying the last drop, the excess DNA was removed from the biosensor by rinsing its surface with deionized water. The response reproducibility was already tested and confirmed in our previous work [18] This method of sensor preparation enabled the complete surface coverage with DNA and prevented non-specific binding of ERL.

2.4. Voltammetric measurements

CV, DPV and SWV were performed for voltammetric measurements. CV was conducted in the potential range between -1.6 V and $+1.6$ V (vs Ag/AgCl, 3 M KCl), starting from 0 V, towards both positive and negative potential, with scan rate ranging from 0.01 V s $^{-1}$ to 0.10 V s $^{-1}$ and step potential of 0.005 V. The conditions used for performing DPV were: step potential 0.005 V, modulation amplitude 0.05 V, modulation time 0.05 s and interval time 1 s. The SWV was performed by using the following conditions: frequency 25 Hz, step potential 0.001 V, effective scan rate of 0.025 V s $^{-1}$ and pulse amplitude of 0.005 V.

The DPV and SWV voltammograms were baseline-corrected with moving average application with step windows of 0.005 V. While the values for peak current were determined from the original voltammograms, for graphical representation of peaks, voltammograms were smoothed using a Savitzky-Golay smoothing algorithm (level 4). All the analysis were performed using the GPES 4.9 software.

2.5. Molecular docking

Besides voltammetric studies, *in silico* analysis of ERL interacting with DNA has been done. Initially, virtual docking has been performed in order to generate a possible ERL-DNA complex. A similar *in silico* study was also performed by Chen et al. [14], which stated that ERL binds by minor groove binding. However, to further explore the possibility of docking results, docking has been performed into both minor groove and as an intercalator. Lapatinib (LAP), an epidermal growth factor receptor type 2 (HER2/ERBB2) and epidermal growth factor receptor (HER1/EGFR/ERBB1) tyrosine kinases inhibitor [19], whose interaction with DNA has previously been examined in voltammetric and spectroscopic [12] studies, has also been used in our studies as a reference compound.

Prior to docking, for geometry optimization of ligands, Gaussian 09 W software [20] was used. The structures of ERL and LAP have been subjected to sequential semi-empirical PM3 and DFT B3LYP (d, p) energy minimization.

We have used two different X-ray structures of double stranded DNA sequences obtained from PDB database (<https://www.rcsb.org/>), pdb: 1BNA for the minor groove binding, and pdb: 2D55 for intercalation. The docking was performed with GOLD 2022.2. software (<https://www.ccdc.cam.ac.uk/solutions/software/gold/>).

Earlier studies performed by Srivastava et al. [21] confirmed reliable results obtained by use of GOLD software for exploring nucleic acid ligand complexes. The binding pocket was defined within 8.0 Å radius of the central nucleic acid, while GoldScore scoring function was used for ranking the predicted poses. The visualization was performed by Discovery Studio Visualizer 2021.

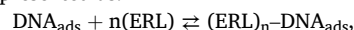
2.6. Molecular dynamics

To further explore the interaction of ERL with DNA, the complexes obtained from the docking studies were subjected to molecular dynamics simulation (MD). The generation of input files was performed on CHARMM-GUI server (<https://www.charmm-gui.org/>). The complexes were solvated into rectangular box of TIP3P model water molecules with 10 Å edge distances. Sodium and chloride ions were added with Monte Carlo method for the system neutralization and final concentration of 0.15 M was obtained. CHARMM36m [22] forcefield was used for generation of topology and parameter files, while for the DNA, and CGenFF [23] was used for the generation of topology and parameter files for ERL and LAP. Minimization (10000 steps) was performed prior to simulations. The equilibration NVT (constant number of atoms, volume, and temperature) run was performed for 250 ps, and the system was heated to 310.15 K. Afterwards, the 50 ns production NPT (constant number of atoms, pressure, and temperature) simulation has been run, while 1–4 scaling and distance related parameters for switching, cut-off and pair-list distances were set to 10 Å, 12 Å and 16 Å, respectively, for the evaluation of the Coulombic and van der Waals interactions. Periodic Boundary Conditions were used for the simulations, and for the long-range electrostatic interactions the particle mesh Ewald (PME) method was used. Langevin temperature coupling with a friction coefficient of 1 ps $^{-1}$ was used for controlling the temperature, while Langevin-piston method (Nosé-Hoover Langevin piston pressure control) with a piston period of 50 fs and a piston decay of 25 fs was used to control the pressure. The simulations were performed by using NAMD 2.14 software and necessary analysis by VMD 1.9.3 software (<https://www.ks.uiuc.edu/>).

3. Theory/calculation

3.1. Calculation of Binding constant

The equilibrium reaction for formation of ERL-DNA complex can be represented as:



from which the equilibrium constant of complex forming can be given by Eq. (1):

$$K = \frac{[(\text{ERL})_n\text{—DNA}_{\text{ads}}]}{[\text{DNA}_{\text{ads}}][\text{ERL}]^n} \quad (1)$$

in which n represents the molar relation of ERL binding to the molar quantity of DNA bases.

Based on the overall Hill cooperativity model [24] the fraction of ERL bound to adsorbed DNA (f) is correlated with dissociation equilibrium constant (K_d), and accordingly with association binding constant ($K = K_d^{-1}$):

$$f = \frac{[(\text{ERL})_n\text{—DNA}_{\text{ads}}]}{[(\text{ERL})_n\text{—DNA}_{\text{ads}}]_{\text{max}}} = \frac{[\text{ERL}]^n}{[\text{ERL}]^n + K_d^n} \quad (2)$$

Since the interaction “partners” for ERL are the base pairs of DNA, it follows that $[(\text{ERL})_n\text{—DNA}_{\text{ads}}]_{\text{max}}$ is the maximum concentration of base pairs that can be complexed, and $[(\text{ERL})_n\text{—DNA}_{\text{ads}}]$ is the concentration of base pairs that are complexed at given ERL concentration. Therefore, the difference between these concentrations represents the concentration of free DNA base pairs available for ERL binding:

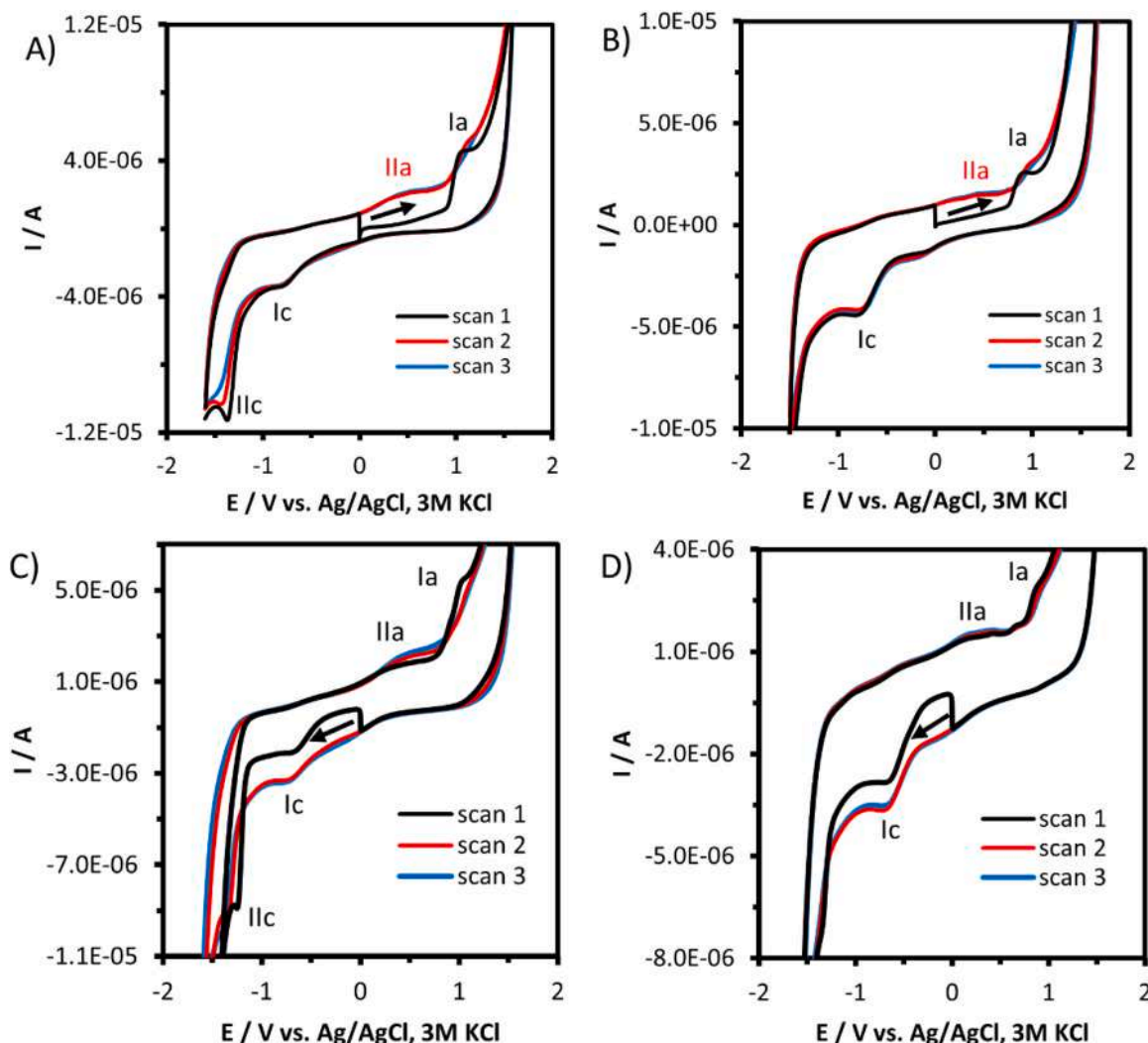


Fig. 2. The CVs of 2×10^{-4} M ERL recorded from 0.0 V in the: positive potential direction at A) pH 4.64 and B) pH 6.94; and negative potential direction at C) pH 4.64 and D) pH 6.94; first (—), second (—) and (—) third scan; $\nu = 50 \text{ mV s}^{-1}$.

$$[\text{DNA}_{\text{ads}}] = [(\text{ERL})_n - \text{DNA}_{\text{ads}}]_{\text{max}} - [(\text{ERL})_n - \text{DNA}_{\text{ads}}] \quad (3)$$

The voltammetric peak currents, obtained under the given experimental conditions with DNA layer adsorbed at the electrode surface and incubated in solutions of different ERL concentration, are given by the relations:

$$\begin{aligned} I_{\text{p-complex}} &= k \bullet n \bullet [(\text{ERL})_n - \text{DNA}_{\text{ads}}] \text{ and } I_{\text{p-DNA}} \\ &= k \bullet n \bullet [(\text{ERL})_n - \text{DNA}_{\text{ads}}]_{\text{max}} \end{aligned} \quad (4)$$

The Eq. (2) can be transformed into the expression:

$$\left(\frac{f}{1-f} \right) = K^n \bullet [\text{ERL}]^n \quad (5)$$

By combining Eqs. (2), (4) and (5) the following expression can be derived:

$$\log \left[\frac{I_{\text{p-complex}}}{I_{\text{p-DNA}} - I_{\text{p-complex}}} \right] = n \log K + n \log [\text{ERL}] \quad (6)$$

where K is the binding constant, $I_{\text{p-DNA}}$ and $I_{\text{p-complex}}$ represent deoxy-adenosine oxidation peak currents, dA (obtained at pH 4.6) in the absence and in the presence of ERL bound to ct-DNA, respectively. Based on the Eq. (6) the association binding constant K and the value of n can be calculated from the intercept and slope of the plot: $\log [I_{\text{p-complex}}/I_{\text{p-DNA}} - I_{\text{p-complex}}]$ vs. $\log [\text{ERL}]$.

DNK - $I_{\text{p-complex}}$) vs. $\log [\text{ERL}]$.

3.2. Binding free energy calculations with MM/PBSA method

Obtained NAMD trajectories were used to calculate binding free energy using MM/PBSA method. Prior to performing the calculation, all the solvent molecules and charged ions have been deleted from the MD trajectories. The snapshots from last 1 ns of the trajectories were used for the binding free energy calculation.

The calculation was performed with CaFE 1.0 plugin [25] in VMD software. The method is consisted of three component - MM (molecular mechanics for gas-phase energy - ΔG_{gas}), PB (Poisson-Boltzmann for polar solvation energy - $\Delta G_{\text{sol}}^{\text{polar}}$) and SA (surface area for nonpolar solvation energy - $\Delta G_{\text{sol}}^{\text{nonpolar}}$). The gas-phase energy difference of complex and separated DNA and ligand is calculated by NAMD software, while APBS 3.4.1 software (<https://server.poissonboltzmann.org/apbs>) was used for polar solvation energy calculation. The interior dielectric constant was set to 4 and exterior dielectric constant to 80 for calculating the PB term. Afterwards the nonpolar solvation energy is calculated by VMD software. The equation for obtaining the binding free energy is:

$$\Delta G_{\text{bind}} = \Delta H - T\Delta S = \langle \Delta G_{\text{gas}} + \Delta G_{\text{sol}}^{\text{polar}} + \Delta G_{\text{sol}}^{\text{nonpolar}} - T\Delta S \rangle \quad (7)$$

The CaFE 1.0 plugin excludes the entropy term from calculation due

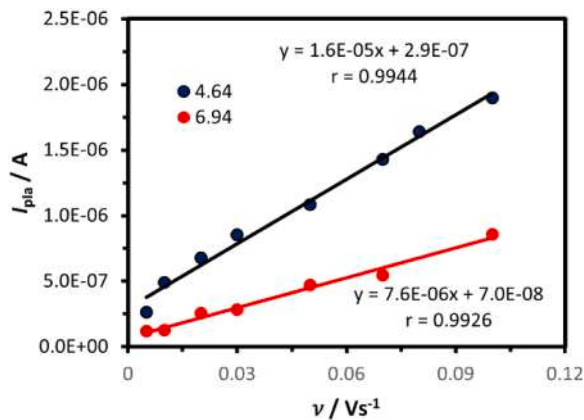


Fig. 3. The effect of the scan rate on the oxidation Ia peak current; pH = 4.64 (—), pH = 6.94 (—).

to inaccuracy of approximations.

4. Results and discussion

4.1. Cyclic voltammetry of ERL at bare GCE

Solutions of 2×10^{-4} M ERL in acetate buffer pH 4.64 and phosphate buffer pH 6.94 were used for obtaining information about oxidation and reduction behavior of ERL with CV. Three successive scans were performed, always starting from 0.0 V, in order to establish and to distinguish the independence between oxidation and reduction processes, first towards positive limit of +1.6 V and then backwards to -1.6 V, and second in opposite direction (initially towards negative, and after towards positive potential limit).

In the first scan performed from 0.0 V towards positive E values (Fig. 2 A and B - black line), one anodic peak (Ia) was detected in solutions of both pH, located at $E_{pia} = 1.035$ V at pH 4.64 and at $E_{pia} = 0.879$ V at pH 6.94. Two cathodic peaks were detected in pH 4.64 solution, Ic at $E_{pic} = -0.771$ V and Ilc at $E_{pic} = -1.357$ V (Fig. 2A), while at pH 6.94 only the first described cathodic peak (Ic) was detected at $E_{pic} = -0.762$ V (Fig. 2B).

When potential was scanned from 0.0 V towards negative E values (Fig. 2 C and D), two cathodic peaks were exhibited at pH 4.64 in the first scan: Ic at $E_{pic} = -0.664$ V and Ilc at $E_{pic} = -1.235$ V (Fig. 2C). In the same scan at pH 4.64, two anodic peaks were detected: peak Ia at $E_{pia} = 1.035$ V and new peak IIa at $E_{pia} = 0.283$ V. At pH 6.94 (Fig. 2D) only one cathodic peak (Ic) at $E_{pic} = -0.630$ V, and two weakly expressed anodic peaks at less positive potentials compared to more acidic conditions, were observed.

It should be noted that the peak IIa was present in the first CV scan only when recorded under the conditions presented in Fig. 2 C and D but was absent from the first scan under the conditions presented at Fig. 2 A and B. This peak existed in all other successive scans and could be the consequence of the oxidation of the ERL reduction product formed at electrode surface when sufficiently negative potential was previously applied. The height of the peak IIa slightly increased in the second and third scan at both pH values investigated.

Analyzing the second and third scan, it can be noticed that all other peaks (Ia, Ic and Ilc) were present, but with a reduced peak current. Based on these results it can be assumed that described oxidation and reduction processes involve adsorption of the active species on the electrode surface.

According to the shape of the peaks, and the lack of the correspondent signal in the backward scans, it can be concluded that all mentioned oxidation and reduction processes were irreversible.

In order to further explore ERL oxido-reduction processes, voltammograms were recorded with the variation of scan rate with two

Table 1 Regression equations and correlation coefficients of the $I_p = f(\omega)$, $I_p = f(\nu^{1/2})$ and $\log I_p = f(\log \nu)$ linear dependences, at pH 4.64 and at pH 6.94.

pH	Conc. (M)	Peak	$I_p / A = f(\nu / V s^{-1})$	r	$I_p / A = f(\nu^{1/2} / V^{1/2} s^{-1/2})$	r	$\log I_p = f(\log \nu)$	r
4.64	2×10^{-4}	Ia	$1.636 \times 10^{-5} \nu + 2.949 \times 10^{-7}$	0.9944	nonlinear	/	$0.625 \log \nu - 5.113$	0.9950
		Ic	$7.110 \times 10^{-6} \nu + 2.265 \times 10^{-7}$	0.9792	$2.795 \times 10^{-6} \nu^{1/2} + 1.460 \times 10^{-9}$	0.9828	$0.489 \log \nu - 5.568$	0.9803
		Ilc	nonlinear	/	$1.780 \times 10^{-6} \nu^{1/2} + 1.492 \times 10^{-7}$	0.9519	$0.352 \log \nu - 5.782$	0.9536
		Ia	$3.473 \times 10^{-6} \nu - 1.311 \times 10^{-8}$	0.9926	nonlinear	/	$0.982 \log \nu - 5.584$	0.9848
6.96	2×10^{-6}	Ic	$1.616 \times 10^{-6} \nu + 3.225 \times 10^{-8}$	0.9884	$6.436 \times 10^{-6} \nu - 2.480 \times 10^{-8}$	0.9804	$0.592 \log \nu - 6.171$	0.9792
		Ia	$7.600 \times 10^{-6} \nu + 6.976 \times 10^{-8}$	0.9926	nonlinear	/	$0.667 \log \nu - 5.477$	0.9814
		Ic	$3.726 \times 10^{-5} \nu + 2.574 \times 10^{-7}$	0.9982	nonlinear	/	$0.684 \log \nu - 4.815$	0.9957
		Ia	$1.310 \times 10^{-6} \nu - 7.309 \times 10^{-9}$	0.9883	nonlinear	/	$0.717 \log \nu - 6.228$	0.9477
	2×10^{-6}	Ic	$3.025 \times 10^{-6} \nu + 8.488 \times 10^{-8}$	0.9921	$1.163 \times 10^{-6} \nu - 2.089 \times 10^{-8}$	0.9851	$0.539 \log \nu - 5.926$	0.9763

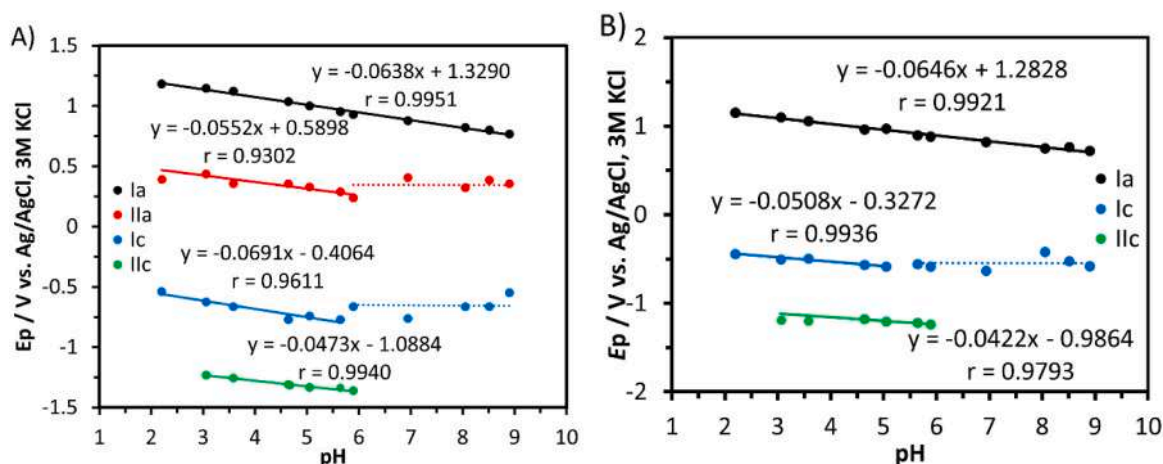


Fig. 4. The influence of pH on the peak potentials of 2×10^{-4} M ERL obtained by A) CV; and B) DPV; ● – peak Ia, ● – peak IIa, ● – peak Ic and ● – peak IIc.

different ERL concentration, high of 2×10^{-4} M, and low of 2×10^{-6} M. The scan rate studies [26–28] were performed at pH 4.64 and 6.94 between 5 and 100 mVs^{-1} .

The effect of potential scan rate (v) on the peak current monitored for 2×10^{-4} M ERL anodic Ia peak at both pH 4.64 and 6.94 gave linear dependence of I_p vs. v plot (Fig. 3, Table 1), what is characteristic for adsorption-controlled processes. Besides that, the slope values over 0.6 of $\log I_p$ vs. $\log v$ dependences confirm the above conclusion [26].

The reduction of 2×10^{-4} M ERL at pH 6.94 could only be investigated in a narrower scan rate range (10–80 mVs^{-1}), due to the distorted shape of the peaks with the increase in v . The cathodic Ic peak, at pH 4.64, manifested diffusion-adsorption mixed controlled process. This can be concluded since both dependences, I_p vs. v and I_p vs. $v^{1/2}$, showed approximately same linear plots with correlation coefficients (r) being around 0.98, and the slope of $\log I_p$ vs. $\log v$ plot being 0.49. The obtained slope value coincides with the theoretical value of 0.5 that represents the limiting value for the criterion that distinguishes between diffusion and adsorption nature of the process [26]. At pH 6.94, on the other hand, adsorption became predominant, as indicated by linear I_p vs. v dependence, and $\log I_p$ vs. $\log v$ slope of 0.684. All mentioned regression equations along with correlation coefficients are listed in Table 1, and the corresponding plots are presented in Fig. S1, in the Supplementary Material.

In order to fully understand the electrochemical behavior of ERL, a scan rate study was performed with lower ERL concentration of

2×10^{-6} M. With this low concentration, the increase of peak currents could be followed only at scan rates below 70 mVs^{-1} , while at higher rates the current levels off. As shown in Table 1, the adsorption effect was confirmed for oxidation process of ERL, and in the case of reduction peak (Ic), adsorption nature is slightly more pronounced compared to higher concentration of ERL. The corresponding plots are presented in Fig. S2, in the Supplementary Material.

The second cathodic peak (IIc) occurs at very negative potentials only at pH 4.64. Since it occurs close to the potential of oxygen discharge and poorly pronounced, precise measurement of the peak height was difficult to perform. That is the reason why all mentioned dependencies did not show high correlation coefficient (Table 1), but still, the results suggest that the process is primarily diffusion controlled [28]. The second cathodic peak cannot be detected at low ERL concentration of 2×10^{-6} M.

4.1.1. The effect of pH on ERL redox reactions

The analysis has been performed in different buffer solutions (pH range between 2.0 and 9.0) in order to track changes of I_p and E_p across different pH values of medium. The peak Ia occurs in all pH values, shifting from +1.2 V to +0.8 V when the pH value increases. The peaks IIa and Ic can be detected at all pH values as well, with peak potentials notably shifting in acid solutions, but without a significant change of potential in neutral and basic medium. The absence of a change in the potential of the peak under these conditions can be explained by the fact

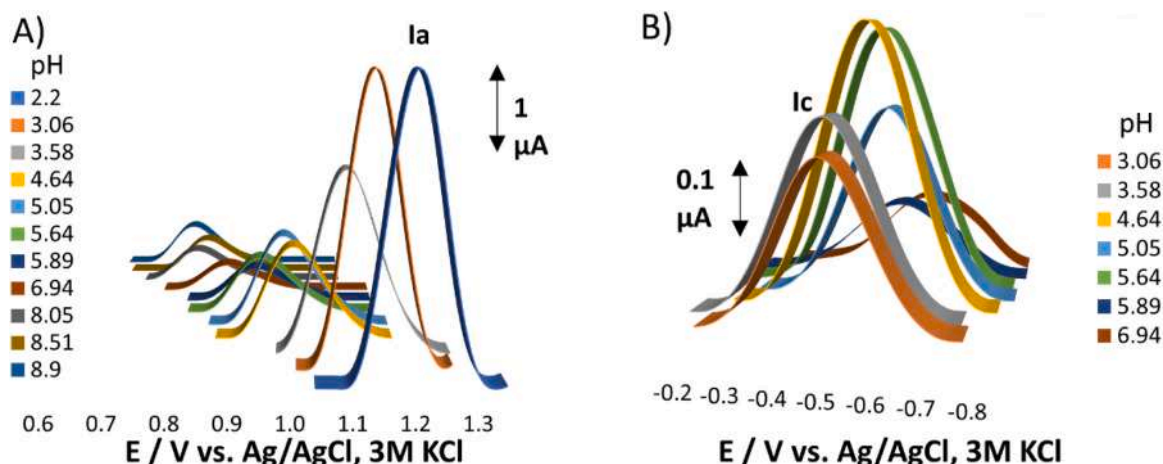


Fig. 5. 3D plot of selected DP voltammograms of 2×10^{-4} M ERL recorded at different pH: A) oxidation peak Ia; B) reduction peak Ic.

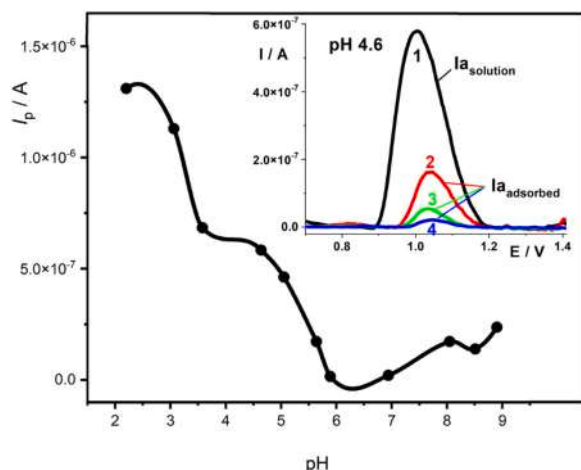


Fig. 6. Plot of I_p vs. pH for the oxidation peak Ia by SWV; Inset: Peak Ia obtained by SWV at pH 4.64 of 2×10^{-4} M ERL in solution (1), and after 30 min of adsorption from the 2×10^{-4} M ERL (2), 2×10^{-5} M ERL (3), and 2×10^{-6} M ERL (4) solution.

that protons do not participate in redox processes, but only electron transfer occurs. The peak IIc occurs in narrow pH range 3.0 – 6.0, with a change of peak potential towards more negative values with the increase of pH. The effect of pH on all ERL CV peaks potential is presented in Fig. 4A.

For the Ia peak, the slope of 63.8 mV/pH (obtained across the whole investigated pH region), which is close to the theoretical value of 59 mV/pH [27], indicates that an equal number of protons and electrons is involved in the rate determining step of the oxidation process. Although this linear dependence was obtained with a high correlation coefficient ($r > 0.99$), a more detailed analysis can distinguish two separate linear segments (Fig. S3, in the Supplementary Material). The values of the slopes of these segments, 71.5 mV/pH and 53.4 mV/pH respectively, confirms the participation of the same number of electrons and protons in both pH areas. From the value of their cross section, $pK_a = 5.8$ can be deduced, what is in good agreement with the value reported for ERL in

the literature ($pK_a = 5.4$ [29]).

The Ia peak potential showed pH dependence only in acid media. Since the shift of the peak potential per pH unit is 55 mV/pH, it suggests that in this oxidation process equal number of protons and electrons is transferred as well. In neutral and basic solutions, no proton transfer is involved due to the lack of pH influence on the E_{pIa} .

The cathodic peak Ic showed results qualitatively like anodic peak Ia. The slope of the E_p vs. pH dependence is 69 mV/pH ($pH < 6.0$) and no pH effect at $pH > 6$, which again refers to equal number of protons and electrons involved in the reduction process in acid medium, and no proton transfer in the neutral and alkaline solutions. Results of $\Delta E_p/\Delta pH$ refer that the reduction process characterized with IIc, as in the previous cases, consumes an equal number of protons and electrons (E_{pIc} vs. $pH \sim 47$ mV/pH; $3.0 < pH < 6.0$).

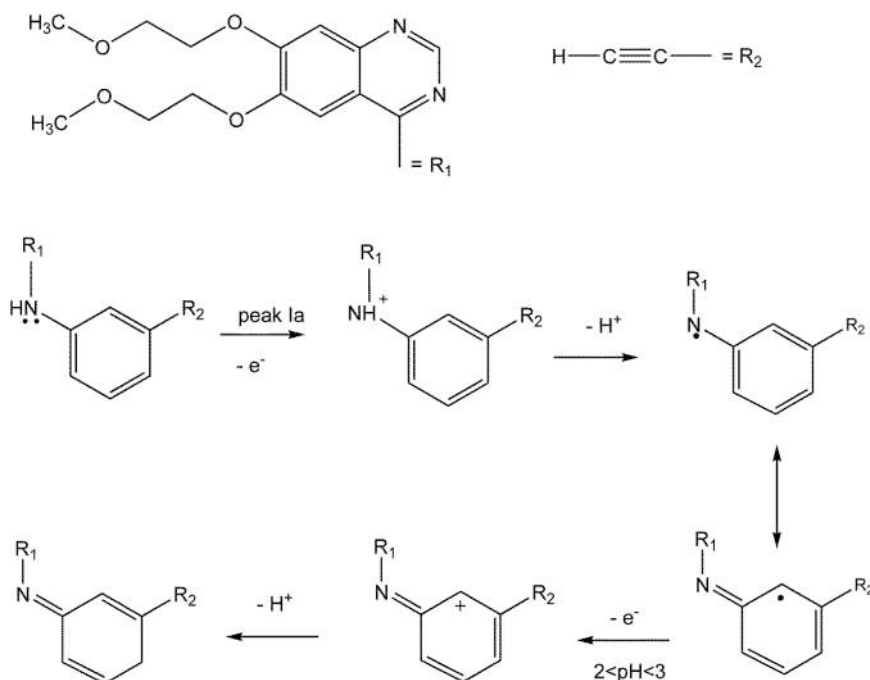
The difference between peak potential and half-height peak potential was calculated across the examined pH region, for all investigated peaks. Considering the equation for irreversible electrode processes [30]:

$$|E_p - E_{p1/2}| = \frac{47.7}{\alpha_n n} \quad (8)$$

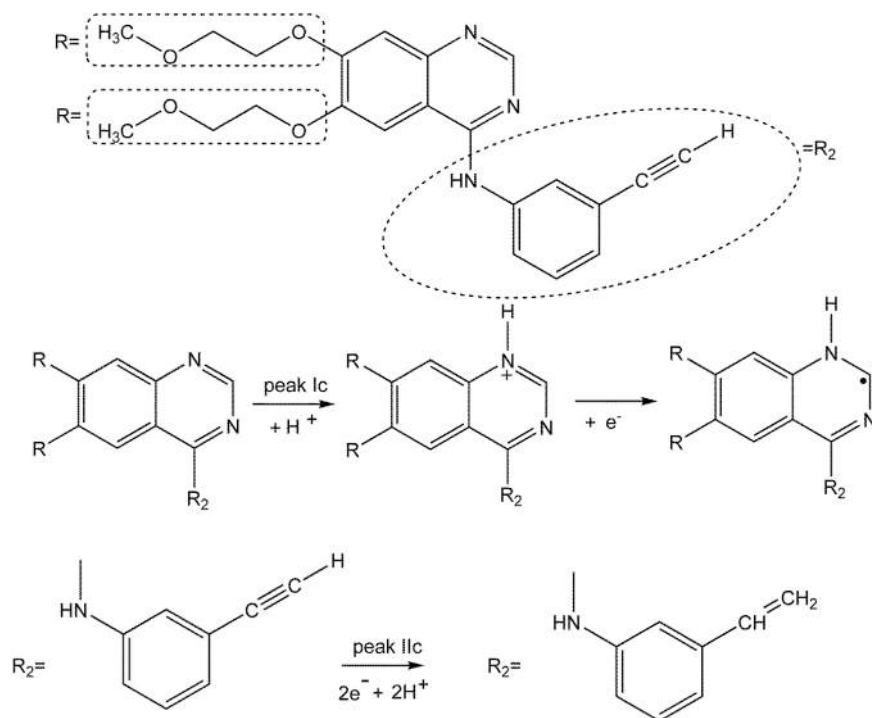
where α_n is the charge transfer coefficient and n the number of electrons transferred in the rate determining step, and comparing with difference between peak potential and half-height peak potential experimentally obtained, the number of exchanged electrons can be determined.

The calculated values for peak Ia was 64 mV (at pH 4.6) and 70 mV (at pH 7.0), while the peaks Ic and IIa were significantly wider and consequently showed $E_p - E_{p1/2}$ between 100 and 120 mV (even more in alkaline solutions). Bearing in mind that for an irreversible process the transfer coefficient value should be ≤ 1 (actually between 0.3 and 0.7), it follows that the rate determining step should involve transfer of one electron in the all mentioned processes. In acid medium, $pH \leq 3$, difference between peak potential and half-height peak potential for peak Ia showed less values (30 – 50 mV), indicating dismission of two electrons, what is in accordance with much higher peak intensities under these conditions. In the case of IIc peak, the potential corresponding to its width was less than 40 mV, indicating two electrons involved in reduction.

The peak current for all anodic and cathodic peaks in different pH media is shown in Fig. S4. in the Supplementary Material.



Scheme 1. Proposed oxidation mechanism of ERL.



Scheme 2. Proposed reduction mechanism of ERL.

4.2. DPV and SWV of ERL at bare GCE

The electrochemical behaviour of ERL was also studied by DPV in the previously described pH range. When scanning the potential in positive direction, one anodic peak can be detected across the whole examined pH region, with E_p shifting from +1.2 V to +0.7 V (Fig. 5A). This peak corresponds to Ia peak previously described in analysis of CV scans. The relationship of E_p vs. pH (Fig. 4B) was linear: $E_{p\text{Ia}} = -0.0646 \text{ pH} + 1.2828$, with the slope close to theoretical value, confirming that process involves the equal number of protons and electrons. The second oxidation peak IIa has not been detected.

DPV scans performed towards negative potentials, showed two reduction peaks. The first one, with E_p shifting from -0.45 V to -0.64 V in different buffers, corresponds to Ic peak (Fig. 5B). The dependence of E_p in the function of pH (Fig. 4B) is linear in pH range 2.2 - 6.0 ($E_{p\text{Ic}} = -0.0508 \text{ pH} - 0.3272$). According to the obtained $\Delta E_p / \Delta \text{pH}$ value, the participation of an equal number of electrons and protons is confirmed.

With the second cathodic peak IIc, which can be detected in pH range 3–6, somewhat minor shift of peak potentials was observed (47 mV/pH), but still enough to indicate the participation of equal number of electrons and protons.

The effect of pH on the DPV peaks current is summarized in Fig. S5 in the Supplementary Material,

The half peak width ($W_{1/2}$) was analysed in order to obtain insight into the number of electrons involved in the oxidation and reduction processes. As already known [10] irreversible systems have broader peaks comparing to reversible, therefore the calculated $W_{1/2}$ values of about 120 mV and 200 mV for Ia and Ic peaks respectively, confirm the statement previously established by CV results, that one electron is exchanged in both processes. As already noticed with CV, Ia DPV peaks obtained in acid solutions ($\text{pH} \leq 3$) were sharper with $W_{1/2} \sim 80\text{--}90$ mV indicating possible transfer of two electrons under these conditions. The same conclusion about the consumption of two electrons was made for the peak IIc based on the $W_{1/2}$ value which was in the range 45–65 mV.

As with CV results earlier, the effect of pH on the DPV Ia peak potential has been studied in detail resulting in the $\text{pK}_a = 5.6$ (Fig. S3, in

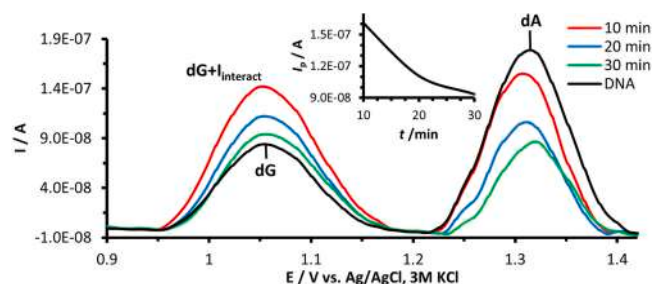


Fig. 7. Baseline corrected SWV voltammograms of ct-DNA biosensor incubated in 2×10^{-5} M ERL solution for different time of incubation.

the Supplementary Material), therefore indicating the even better agreement with literature data.

The SWV was also used for analyzing the electrochemical behavior of ERL. The results, similar to DPV were obtained and confirmed the irreversibility of electrode processes, as well as the number of exchanged electrons and protons. Special attention was paid to SW oxidation peak Ia. The effect of pH on the peak Ia potential is given by the equation: $E_{p\text{Ia}} = -0.0623 \text{ pH} - 1.3057$; $r = 0.9928$; and the influence on the height of the Ia peak is presented at Fig. 6. Although the maximal peak current was obtained at $\text{pH} \leq 3$, the best peak stability and reproducibility was obtained in the range $3.5 \leq \text{pH} \leq 5.0$. At $\text{pH} > 5.5$, ERL peak by SWV was rather poorly obtained. This oxidation process was previously identified as adsorption-controlled, and it was checked and confirmed by recording the SW voltammogram of adsorbed ERL. For this purpose, GCE was immersed in solutions of different concentrations of ERL for 30 min and then transferred to the electrochemical cell with acetate buffer pH 4.64. As seen from the Fig. 6 Inset, the adsorbed ERL showed lower peak currents and slightly more positive potentials for peak Ia compared to the peak obtained directly from the solution without adsorption. These observations were expected for a compound easily adsorbed and when the transfer technique was applied.

Table 2

Normalized peak currents (d(dA)%) and peak potentials ($E_{p, dA}$) obtained after different time of incubation of ct-DNA biosensor in 2×10^{-5} M ERL solution.

Time (min)	d (dA) (%)	$E_{p, dA}$ (V)	ΔE_p (V) ($E_{p-DNA} = 1.315$ V)
10	87.50	1.308	- 0.007
20	60.33	1.311	- 0.004
30	50.76	1.319	0.004

4.3. Redox mechanism

According to CV and DPV results, the oxidation process of ERL (characterized with Ia peak) requires transfer of one proton and one electron, except in an acidic medium, where oxidation takes place with the exchange of 2 electrons and 2 protons. It may be assumed that the oxidation of ERL is related to the secondary aromatic amine group, which upon the deprotonation and losing of an electron yields nitrenium radical. In acidic medium, this process may be continued by leaving off the second electron and the formation of carbocation, and its stabilization to a conjugated imine. The proposed mechanism, which is in accordance with already published literature data [12,31], is presented at the Scheme 1.

The reduction of ERL presented by peak Ic, as suggested by both CV and DPV results, was proceeded with the uptake of one electron and one proton by the nitrogen atom at quinazoline ring system. The peak IIc is most likely a consequence of the reduction of the terminal acetylenic group in the R₂ side chain, which takes place at pH < 6.0, with the consumption of two electrons and two protons according to the mechanism shown in Scheme 2.

4.4. ERL-DNA interaction

For exploring the interaction between ERL and ct-DNA, the multi-layer electrochemical ct-DNA biosensor was used and SW voltammetry was performed. The application of SWV was previously used by our team for investigation of DNA oxidation studies and was proven to perform better comparing to DPV because of higher speed, better repeatability, better resolved voltammograms and reduced surface poisoning [18,32]. The measurements were performed in acetate buffer pH 4.64, due to higher and better shaped DNA oxidation peaks compared to neutral phosphate buffer. The stability of ct-DNA helix at this pH has already been confirmed by other researchers [33] and our team [18].

To follow the interaction, the DNA biosensor was incubated in ERL

solutions of different concentrations in acetate buffer at pH 4.64 for 30 min. As a control, the ct-DNA biosensor incubated in acetate buffer at pH 4.64 for 30 min was used. The voltammogram obtained with control ct-DNA biosensor showed two oxidation peaks: at + 1.05 V representing the oxidation of deoxyguanosine (dG) and at + 1.31 V (Fig. 7) representing the oxidation of deoxyadenosine (dA) [32].

The interaction of small molecule with surface-immobilized DNA caused changes to oxidation peaks dG and dA due to changes in DNA morphology [34]. Furthermore, with multi-layer biosensor, the complete coverage of the electrode surface with DNA was accomplished with the aim to avoid the non-specific adsorption of interacting molecules, so the biosensor response comes only from a compound that is incorporated into the DNA layer, with no contribution from the free compound in the solution [34]. In present case, after the incubation of biosensor in ERL solution, two SWV peaks were registered: (dG+I_{interact}) due to the superimposed oxidation currents of deoxyguanosine and ERL inside the DNA layer, and dA peak. Due to the complex nature of the first mentioned peak, it cannot be used to evaluate the interaction, and therefore only the changes in dA peak height and position were informative.

The initial investigation was done with the intention of establishing the optimal incubation time. In order to do that, the ct-DNA biosensor was incubated in 2×10^{-5} M ERL solution for 10, 20 and 30 min, and a reduction of the peak was registered with increasing incubation time (Fig. 7).

The decrease of dA current has been represented as normalized peak current, which represents a percentage of signal change, and has been calculated by following equation:

$$d(dA) \% = \frac{I_{p-\text{complex}, dA}}{I_{p-DNA, dA}} \times 100 \quad (9)$$

in which d(dA) represents the normalized peak value, $I_{p-DNA, dA}$ and $I_{p-\text{complex}, dA}$ represent dA peak currents in the absence and in the presence of bound ERL to the ct-DNA, respectively. The normalized peak current values and the change of dA peak potentials for different incubation times of ct-DNA biosensor incubated in 2×10^{-5} M ERL solution are shown in Table 2.

The decrease of I_p between 20 and 30 min of incubation time compared to the decrease between 10 and 20 min is much smaller, and the d(dA) value at 30 min is $\approx 50\%$. This implicates that 30 min was sufficient to give quantitative response of formed interaction between ERL and ct-DNA.

To explore the effect of change in concentration of ERL solutions on

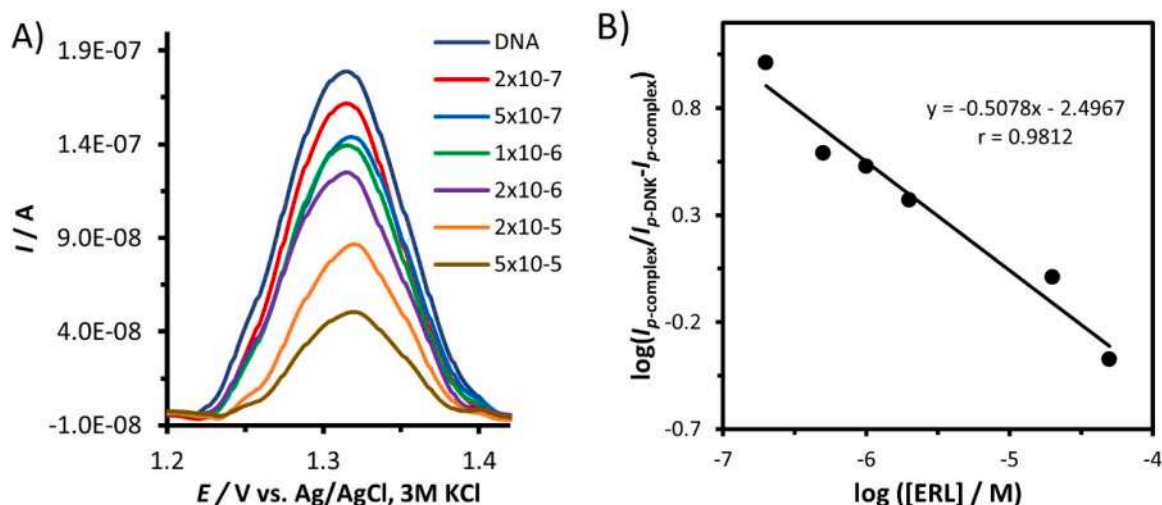


Fig. 8. A) Baseline corrected SWV voltammograms showing dA peak of ct-DNA biosensor incubated in a series of ERL solutions; B) Dependence of $\log \left[\frac{I_{p-\text{complex}}}{I_{p-DNA} - I_{p-\text{complex}}} \right]$ vs $\log [ERL]$ used for ERL-DNA binding constant determination.

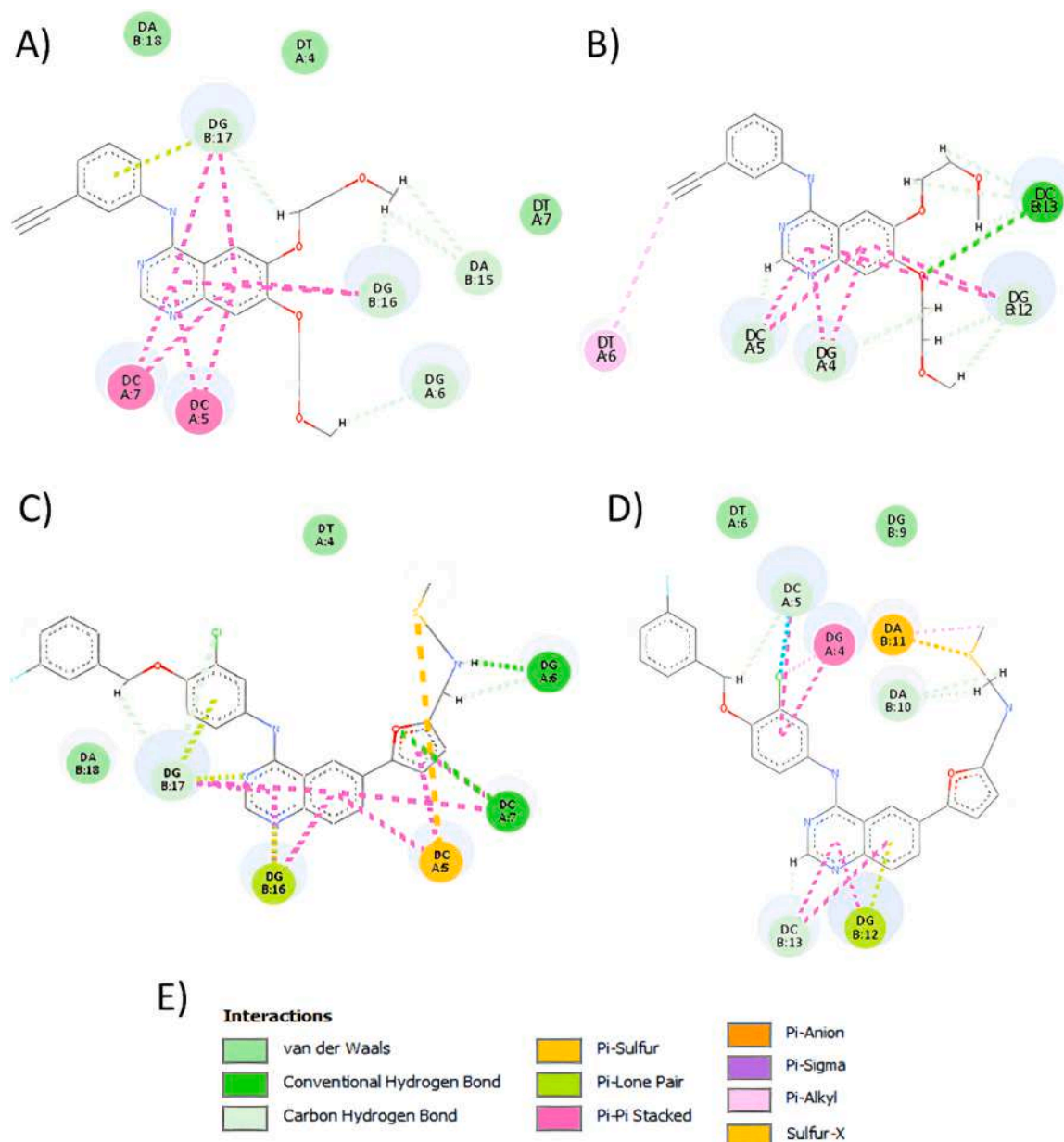


Fig. 9. 2D interactions of A) ERL bound to minor groove (1BNA); B) ERL intercalated (2D55); C) LAP bound to minor groove (1BNA); D) LAP intercalated (2D55) and E) legend.

the interaction of ct-DNA and ERL, a series of solutions with concentration of ERL in the range of 2×10^{-7} M to 5×10^{-5} M was used for 30 min incubation. SWV measurements have shown that when increasing the concentration of ERL in solution, the dA peak height at +1.3 V was decreasing (Fig. 8A) as a direct consequence of ERL binding to ct-DNA and disruption of the oxidation process of deoxyadenosine. The normalized peak current values and the change of dA peak potentials for different ERL concentrations are shown in the Table S1 in the Supplementary Material. The dA peak current reduction up to 30% of the initial value indicates significant interaction with ERL.

The decrease of dA current in the presence of ERL ranged from 2×10^{-7} M to 5×10^{-5} M was further examined in order to calculate the binding constant (K) of ERL to the ct-DNA. Using the Eqs. (1) and (2) (Section 2.1) and having in mind that the concentration of DNA adsorbed on the electrode surface was constant whilst ERL concentration increased during the experiment, K has been obtained from the intercept of the linear dependence: $\log [I_{p-\text{complex}}/(I_{p-\text{DNA}} - I_{p-\text{complex}})] = -2.4967 -$

Table 3
GoldScore values obtained from docking studies.

	ERL	LAP
Minor groove binding (1BNA)	72.75	95.13
Intercalation (2D55)	75.06	86.57

$0.5078 \log [\text{ERL}]$ (Fig. 8B). The calculated value was $K = 8.25 \times 10^4 \text{ M}^{-1}$.

This K value with the small shift of E_p value could implicate a moderately intercalation binding mode of ERL to DNA, and/or possible groove binding [35]. This finding is in agreement with results of lapatinib [12] and ibrutinib [13] binding mechanism. On the other hand, it is inconsistent with the study [15] that reported electrostatic interaction and shift of the DNA oxidation signals after the interaction with ERL. The reason for this difference is probably due to the modification of electrode surface by coating with poly L-methionine.

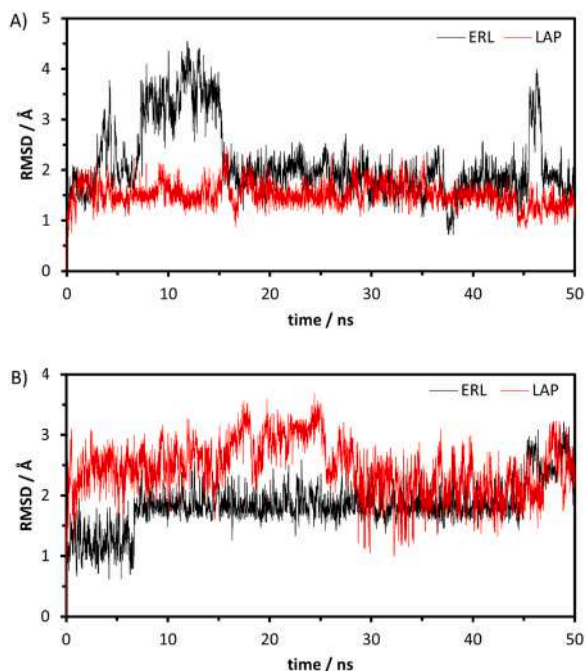


Fig. 10. RMSD during production run of A) nucleic acids from binding site and B) ligands in ERL-DNA and LAP-DNA intercalation complexes.

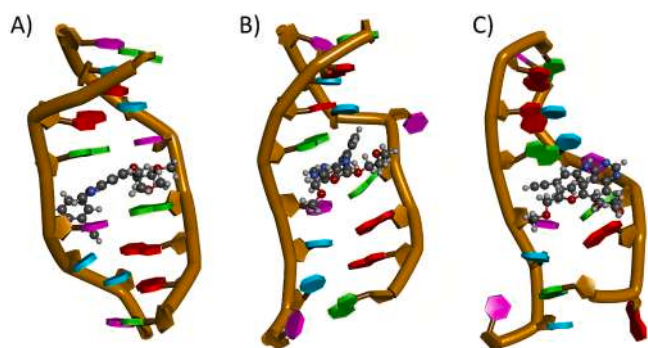


Fig. 11. 3D representation of ERL-DNA intercalation complex A) obtained from docking; B) after 10 ns MD production run; C) after 50 ns MD production run.

The slope of the equation ($n = 0.5078 \approx 0.5$) points out the formation of a 2:1 association complex between ERL and ct-DNA. Based on the obtained binding constant of the complex of the ct-DNA and ERL, the change of Gibbs free energy was calculated by following equation:

$$\Delta G = -RT \ln K \quad (10)$$

The value of $\Delta G = -28.05$ kJ/mol was obtained which describes the stability of the formed adsorbed complex and the spontaneity of the binding process.

4.5. Computational modelling of ERL-DNA interaction

The molecular docking poses of ERL and a reference compound LAP have been obtained in both used DNA structures, implying possibility of both types of interaction. Both ERL and LAP form different interactions with nucleic acids in sequences, and their poses are shown in Fig. 9.

Fig. 9 shows that ERL quinazoline ring forms hydrophobic interactions with examined DNA sequence when docked into minor groove. Benzene ring and both methoxy groups form hydrophobic interactions as well. On the other hand, when intercalated, ERL forms hydrophobic contacts with quinazoline ring, multiple interactions

Table 4

Binding free energies of ERL and LAP.

	ERL	LAP
MM/PBSA (kJ/mol)	− 146.93	− 214.40
Voltammetric (kJ/mol)	− 28.05	− 32.98*

* Calculated based on reported binding constant ($K = 6.03 \times 10^5 \text{ M}^{-1}$) [12] with Eq. 10.

formed with both 2-methoxyethoxy substituents and with the ethynyl group. Oxygen of one methoxyethoxy substituent also forms a hydrogen bond with deoxycytidine (DC B: 13).

LAP when docked to minor groove forms two hydrogen bonds with furane ring (with deoxycytidine DC A: 7) and with secondary amine connected to furane (with deoxyguanine DG A: 6). Chlorobenzene, quinazoline and methylsulfonyl ethyl substituents also form different hydrophobic interactions with the DNA. LAP when intercalated forms multiple hydrophobic contacts involving, quinazoline ring, chlorobenzene ring and methylsulfonyl ethyl substituent.

Obtained GoldScore values (Table 3) suggest that LAP shows higher affinity towards both DNA structures compared to ERL, but further analysis was still necessary.

Complexes of ERL and LAP have been subjected to MD simulations to predict the stability of obtained complexes. It has been shown that both ERL and LAP unbind from DNA minor groove under 10 ns of production time, whereas intercalated ligands stay in bound state with used DNA structure. It has been noted that the DNA structure unwinds during the simulation time, thus for stability of the interaction between ligands and DNA nucleic acids inside binding site are checked for RMSD change. The fluctuation of RMSD values of minor groove binding nucleic acids were higher than 2 Å in some parts of simulation while the ligands were bound to the DNA, and it has been represented in Fig. S6. It can be noticed that at RMSD values of both ERL and LAP binding nucleic acids in intercalation complexes oscillate under 2 Å after 20 ns (ERL crossing the limit in few snapshots) indicating fairly stable interaction between them (Figs. 10 and 11). These results together with voltammetric studies imply that the intercalation could be more dominant way ERL binds to DNA compared to minor groove binding. A higher RMSD value fluctuation of LAP could be due to its bigger structure parts which don't form interactions with DNA and thus move more freely.

The MM/PBSA calculations were performed to acquire information of affinity of ERL and LAP for DNA. The free energy value calculated for ERL is − 146.93 kJ/mol, and − 214.40 kJ/mol for LAP. Higher values compared to experimental values gained in our work and the work made for LAP [12] calculation has been made because snapshots of trajectory parts with stable RMSD fluctuation were used. The difference could also be because one DNA sequence has been used for the MD simulation to explore the nature of non-specific binding of ERL to DNA. However, the ratio of these values (Table 4) is similar to the ratio of experimental values which supports our hypothesis about the type of interaction of ERL with DNA.

5. Conclusions

The tyrosine kinase inhibitor erlotinib is electroactive in the whole investigated pH range (pH 2.0 – 9.0) and undergoes separate processes of oxidation and reduction on GCE. Oxidation of ERL is irreversible adsorption-controlled process which proceeds with the transfer of one proton and one electron, except in an acidic medium, where the exchange of 2 electrons and 2 protons occurs. It is presumed that the oxidation of ERL take place at the secondary aromatic amine group, which upon the deprotonation and losing of an electron yields nitrenium radical. In acidic medium, this process may be continued by leaving off the second electron and the formation of carbocation. The reduction process (presented by peak Ic) in mild acidic medium manifested diffusion-adsorption mixed controlled process, while in neutral

solutions, adsorption became predominant. As suggested by both CV and DPV results, this reduction occurs with the uptake of one electron and one proton by the nitrogen atom at quinazoline ring system. In the acidic solutions, pH < 6.0, the reduction of the terminal acetylenic group in the ERL R₂ side chain occurred as a diffusion-controlled process with the consumption of two electrons and two protons.

The interaction between ERL and DNA was investigated using the multilayer electrochemical ct-DNA biosensor incubated in ERL solutions, and the measurements were performed with SWV. Series of solutions with concentration of ERL in the range of 2×10^{-7} M to 5×10^{-5} M were used to follow the interaction between surface-immobilized DNA and ERL in the solution. Having in mind that multi-layer biosensor provides the complete coverage of the electrode surface with DNA, and the change in biosensor response originates only from ERL incorporated into the DNA layer, the decrease of dA peak height was a direct consequence of ERL binding to ct-DNA and disruption of the oxidation process of deoxyadenosine.

SWV measurements have shown that the increasing of ERL concentration in the solution, caused a further decrease in dA peak height, what was utilized to calculate the binding constant, $K = 8.25 \times 10^4 \text{ M}^{-1}$ of ERL to the ct-DNA. The obtained value, together with the small shift of E_p suggested a moderately intercalation binding mode of ERL to DNA, and/or possible groove binding.

Molecular docking and molecular dynamics analysis were used to verify and support the experimental findings. Molecular docking showed that ERL quinazoline ring forms hydrophobic interactions when docked into minor groove. When intercalated, ERL forms hydrophobic contacts with quinazoline ring, acetylenic group and several interactions with 2-methoxyethoxy substituents. Molecular dynamics analysis predicted the stability of obtained intercalation complexes and further depicted the nature of the interaction between the DNA and ERL. The MM/PBSA method was also used to compare binding energies between ERL and a reference compound LAP.

The results of this research will contribute to the understanding of the mechanism of interaction between erlotinib and nucleic acids.

CRediT authorship contribution statement

Milan Jovanović: Conceptualization, Methodology, Formal analysis, Writing - original draft. **Katarina Nikolic:** Conceptualization, Methodology, Resources, Supervision, Writing - review & editing. **Marija Carapić:** Writing - original draft. **Mara M. Aleksić:** Supervision, Conceptualization, Methodology, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jpba.2023.115560](https://doi.org/10.1016/j.jpba.2023.115560).

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Application of parallel artificial membrane permeability assay technique and chemometric modeling for blood–brain barrier permeability prediction of protein kinase inhibitors

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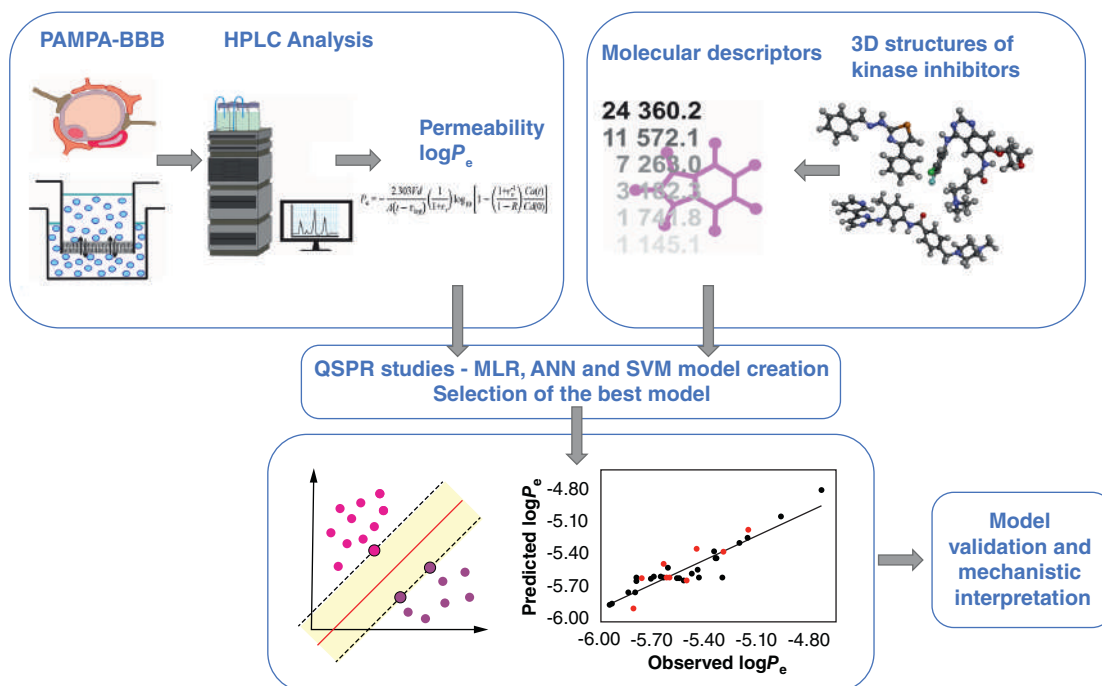
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Aim: This study aims to investigate the passive diffusion of protein kinase inhibitors through the blood–brain barrier (BBB) and to develop a model for their permeability prediction. **Materials & methods:** We used the parallel artificial membrane permeability assay to obtain logPe values of each of 34 compounds and calculated descriptors for these structures to perform quantitative structure–property relationship modeling, creating different regression models. **Results:** The logPe values have been calculated for all 34 compounds. Support vector machine regression was considered the most reliable, and CATS2D_09_DA, CATS2D_04_AA, B04[N-S] and F07[C-N] descriptors were identified as the most influential to passive BBB permeability. **Conclusion:** The quantitative structure–property relationship-support vector machine regression model that has been generated can serve as an efficient method for preliminary screening of BBB permeability of new analogs.

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Keywords: ANN • MLR • PAMPA-BBB • protein kinase inhibitors • QSPR • SVM

Graphical abstract:



The blood–brain barrier (BBB) is a highly selective system composed of non-fenestrated endothelial cells of the brain capillaries connected by much tighter junctions than the peripheral vessels and supported by pericytes in the membrane base and astrocytes [1]. With its complex transport mechanism, the BBB regulates the transport of nutrients, ions and hormones, and prevents neurotoxins or pathogens from entering the brain [2]. The discovery of drugs that can reach the CNS is a very difficult and important phase in the preclinical research of CNS-targeted drugs [3]. In addition, blood–brain penetration testing of drug candidates targeting peripheral tissues is necessary to avoid side effects on the CNS [4].

Protein kinases play an important role in cell regulation and protein phosphorylation in various cellular signaling pathways [5]. Due to numerous physiological functions in different systems and tissues (cardiovascular system, kidney, CNS, etc.), phosphorylation deficiencies can be associated with Type II diabetes, cardiovascular diseases, Alzheimer's disease, other neurodegenerative diseases and cancer [6]. Currently, protein kinases have become one of the most important drug targets with more than 70 approved therapeutics against cancer and inflammation. Approximately a third of global drug discovery efforts target protein kinases [7], while 180 clinical trials of orally administered protein kinase inhibitors are ongoing [8]. Some protein kinases have recently emerged as promising targets for the treatment of neuroinflammation and neurodegenerative diseases such as stroke, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis.

A new concept, known as 'aberrant cell cycle disease' (ACCD) suggests that cancers and neurological disorders share a common mechanism characterized by an 'aberrant re-entry into the cell cycle' [9,10]. This phenomenon involves the activation of kinases/oncoproteins, which leads to the proliferation of tumor cells in cancer and the death of neurons in neurological disorders. Since kinases play an important role in the cell cycle process, kinase inhibitors could have significant potential for the treatment of both cancer and neurological disorders.

Protein kinases are the hallmark of neoplasms due to their role in signaling mechanisms in malignant cell activity, and therefore protein kinase inhibitors play the most important role in cancer chemotherapy. However, one of the limitations is the risk of significant drug–drug interactions, toxicity, off-target effects and resistance. Since most kinase inhibitors are metabolized by cytochrome 450 isozymes there is a high risk of interactions with CYP (CYP3A4) inhibitors/inducers [11].

Apart from primary CNS tumors such as neuroblastoma and glioblastoma, more than 40% of peripheral tumors metastasize to the CNS. However, the discovery of kinase inhibitor for the treatment of brain tumors is very difficult due to the complexity of drug penetration through the BBB and drug distribution in the CNS. Despite

this challenge, several therapeutic candidates targeting CNS protein kinases are currently in various stages of preclinical and clinical development [12,13].

In this study, the BBB permeability of 34 structurally diverse derivatives was investigated, including 15 approved protein kinase inhibitors – afatinib, axitinib, erlotinib, gefitinib, imatinib, crizotinib, lapatinib, nilotinib, nintedanib, ruxolitinib, palbociclib, dabrafenib, vemurafenib, everolimus and fasudil (Supplementary Figure 1). N-desmethyl-imatinib – an impurity of imatinib (defined in the European Pharmacopeia as imatinib EP impurity C) – and RKI-1447 as an experimental ROCK inhibitor were also investigated. New experimental compounds were also investigated to expand the dataset, including 2 synthetic aryl-2-aminopyridine derivatives (compounds **1** and **2**) [14] and 15 pyridyl-based thiazolyl hydrazone and selenazolyl hydrazone derivatives (compounds **3–17**) [15,16], some of which have proven anticancer activity and whose scaffolds are known to exhibit protein kinase inhibitory activity (Supplementary Figure 2).

The development of reliable *in vitro* methods for the screening of drugs that can cross the BBB is of great importance for the early phase of drug development. One of the most efficient and widely used *in vitro* methods to assess BBB permeability is the parallel artificial membrane permeability assay (PAMPA-BBB) [17]. The PAMPA-BBB assay is based on the passive diffusion of test substances through a membrane that mimics the physicochemical environment of the BBB [18,19]. Recent publications continue to rely on the study of PAMPA-BBB to understand the structural features that are important for the passive permeability of various substances through the BBB [20,21]. Despite the limitation of PAMPA-BBB that active or efflux transporters cannot be modeled, PAMPA-BBB is a very useful method because most drugs are absorbed by passive diffusion through the BBB [22]. Compared with cell-based methods, the PAMPA-BBB assay is time and cost effective, reliable, efficient and suitable for high-throughput screening and therefore very useful in the early stages of CNS drug discovery.

Quantitative structure–property relationship (QSPR) studies using various machine learning approaches are applied at various stages of drug discovery [23], by using molecular descriptors to correlate chemical structures with BBB permeability and allow prioritization of compounds with favorable properties for further testing, thereby improving the discovery of more effective CNS drugs.

The PAMPA assays provide screening capabilities by simulating passive diffusion through the BBB, while QSPR modeling provides predictive insights into the molecular features that influence permeability. Therefore, the main objective of this research is to develop chemometric models for the BBB permeability of different types of structurally diverse protein kinase inhibitors and to enable efficient discovery of CNS protein kinases.

This approach, which focuses on both structural diversity and different modes of action, is more useful in drug discovery and provides a flexible tool to identify kinase inhibitor candidates with improved BBB penetration. The results of this research could help advance the development of kinase inhibitors by enabling rapid selection of compounds based on desired BBB permeability, thereby improving the drug discovery process. This chemometric study also provides rapid screening of new candidates and the identification of potential therapeutic options for CNS diseases.

Materials & methods

Chemicals & reagents

Reference compounds that include US FDA-approved kinase inhibitors: afatinib, axitinib, dabrafenib–mesylate, erlotinib–hydrochloride, everolimus, fasudil, gefitinib, imatinib, crizotinib, lapatinib–ditosylate, nilotinib–hydrochloride, nintedanib–esylate, palbociclib, ruxolitinib–phosphate, vemurafenib and compounds that affect CNS like levodopa and haloperidol as well were obtained from Sigma-Aldrich (MO, USA). Imatinib EP impurity C (N-desmethyl imatinib) was obtained from Sigma-Aldrich and RKI-1447 was obtained from Selleck Co. (Shanghai, China). The experimental compounds **1–17** were obtained from V. Savic [14] and T. Todorović/N. Filipović [15,16] research groups.

The physiological phosphate buffer (pH = 7.4) was prepared using sodium chloride (JT Baker, Deventer, The Netherlands), sodium hydrogen phosphate and potassium dihydrogen phosphate (Merck, Darmstadt, Germany).

Alongside the buffer, dimethyl sulfoxide (DMSO) and Tween-80 (both obtained from Sigma-Aldrich) were used for preparing compound solutions. Dodecane (Sigma-Aldrich) and the porcine polar brain lipid (PBL; catalog no. 141101C), purchased from Avanti Polar Lipids, Inc. (AL, USA), were used for membrane preparation.

The PAMPA 96-well plates (MultiScreen-HV, 0.45 μ m, clear, cat. no. MAHVN4510) were purchased from Millipore, Inc. (MA, USA). All solutions prepared for high-performance liquid chromatography (HPLC) analysis were passed through a 0.45 μ m nylon syringe filters (Amast, FL, USA) before analysis.

HPLC grade reagents were used to prepare the mobile phase: acetonitrile (ACN; JT Bakers), disodium hydrogen phosphate (Sigma-Aldrich), orto phosphoric acid, 85% (Sigma-Aldrich), and deionized water (Thermo Electron LED GmbH, Germany, TKA GenPure).

Artificial membrane permeability assay

The PAMPA-BBB model, previously described in literature [19] was used to examine the effective permeability of structurally diverse derivatives. Alongside, permeability of levodopa and haloperidol have also been examined to validate the procedure. Stock solutions of the compounds were prepared in DMSO (5 mg/ml) and were further diluted in physiological phosphate buffer. Tween 80 was added in 5% concentration to ensure good solubility of low soluble compounds in the buffer [24]. Final solutions were obtained in concentration of 25 µg/ml that was used for PAMPA analysis.

The artificial membrane solution was prepared with 20 mg of PBL dissolved in 1 ml dodecane. A total of 4 µl of the solution was used to coat the well plates' filter membrane. The donor wells were filled with 300 µl of the prepared solutions of each tested compound, and the acceptor wells were filled with 300 µl of the phosphate buffer containing 5% Tween-80. Afterward, the acceptor plates were placed on top of the donor plates, thus creating a 'sandwich', covered from the top, with the whole system wrapped up with parafilm. The system was left for 18 h incubation at 25°C. After the incubation, the 'sandwich' was disassembled carefully, and HPLC with a UV detector was used to determine the concentration of the starting solutions as well as each donor and acceptor well. All samples were analyzed in triplicate, and the mean values of concentrations were reported. Equations used for calculating the effective permeability of the tested compounds are (Equation 1 and 2):

$$VaCa(t) + VdCd(t) = VdCd(0) (1 - R) \quad (\text{Equation 1})$$

$$P_e = -\frac{2.303Vd}{A(t - \tau_{lag})} \left(\frac{1}{1 + r_v} \right) \log_{10} \left[1 - \left(\frac{1 + r_v^{-1}}{1 - R} \right) \frac{Ca(t)}{Cd(0)} \right] \quad (\text{Equation 2})$$

in which Va – acceptor well volume – 300 µl; Vd – donor well volume – 300 µl; A – filter area – 0.28 cm²; t – incubation time – 64 800 s; $Ca(t)$ – compound concentration in acceptor wells after incubation; $Cd(t)$ – compound concentration in donor wells after incubation; $Cd(0)$ – compound concentration in donor wells before incubation; R – mole fraction of compound retained by a membrane; r_v – donor volume (300 µl) and acceptor volume (300 µl) ratio – $Vd/Va = 1$; τ_{lag} – time needed for the membrane saturation (approx. 20 min).

High-performance liquid chromatography analysis

The HPLC analysis was performed using Dionex UltiMate 3000 (Thermo Scientific, Germering, Germany) equipped with Diode Array Detector (DAD). Chromatographic analyses were performed on the Poroshell 120 EC-C18, 250 × 4.6 mm (particle size 5 µm) chromatographic column. Column temperature was set on 30°C, and autosampler temperature on 10°C. Injection volume was 20 µl. For most of the compounds, flow rate was 0.8 ml/min, except for compound 2 (1 ml/min). UV detection was performed at 230, 254, 265 and 278 nm. The mobile phase contained a mixture of 10 mM phosphate buffer, pH 2.3 and ACN at ratios varying from 30:70 (v/v) to 90:10 (v/v), respectively.

Dataset preparation

Kinase inhibitor structures set, obtained from Drugbank database [25], has been subjected to hierarchic clustering in DataWarrior 5.5 software [26] based on FragFp (substructure fragment dictionary-based binary fingerprint) to ensure coverage of different chemical structure types of kinase inhibitors used in clinical practice (Supplementary Table 1).

Dominant forms at physiological pH = 7.40 of the prepared dataset compounds were checked in the Marvin Sketch 20.8 program. The geometry of these structures was then optimized within the Gaussian 09W program [27] using the semiempirical PM3 (Parameterized Model revision 3) method and subsequently with the Hartree-Fock/3-21 G method.

Molecular descriptors calculation

DRAGON 6.5 [28] and ADMET Predictor 13.1 [29] software have been used to calculate a set of different geometrical, electronic, topological and physicochemical descriptors for the dataset. Descriptors with low and zero variance have

Table 1. Training and test sets used in creating the quantitative structure–property relationship models.

Training set		Test set
Afatinib	Compound 2	Gefitinib
Axitinib	Compound 4	RKI-1447
Dabrafenib	Compound 5	Ruxolitinib
Erlotinib	Compound 6	N-desmethyl imatinib
Everolimus	Compound 7	Compound 1
Fasudil	Compound 9	Compound 3
Imatinib	Compound 11	Compound 8
Crizotinib	Compound 12	Compound 10
Lapatinib	Compound 14	Compound 13
Nilotinib	Compound 15	
Nintedanib	Compound 16	
Palbociclib	Compound 17	
Vemurafenib		

been removed. They were also checked for collinearity, by which only descriptors with intercorrelation lower than 0.90 were used for the predictive model building. Z-score normalization was used to scale descriptors, so the final set of 910 descriptors was prepared.

Quantitative structure–property relationship modeling

Several methods were applied to obtain multiple QSPR models. The descriptors of the studied compounds were considered as independent variables, while the $\log P_c$ value obtained from the PAMPA study was used as the dependent variable. Three machine learning methods were employed to develop QSPR models – multiple linear regression (MLR), support vector machine regression (SVM) and artificial neural network (ANN). All models were created using STATISTICA 13.6 software [30].

The MLR model assumes a linear relationship between the dependent and independent variables. Stepwise regression was used to select the variables in the MLR model development based on F to add and F to remove criteria. SVM and ANN models were then created with the same selected variables.

The SVM regression modeling was performed to investigate possible nonlinear dependencies of the selected variables with $\log P_c$. Regularization parameter (C), the gamma parameter (γ) and the ϵ -intensive objective function parameter (ϵ) are hyperparameters that were optimized in the search for the optimal model. The cross-validation method was used to tune the hyperparameters. Four kernels were used to search for an optimal support vector regression model: linear, polynomial, RBF (Radial Basis Function) and sigmoid.

ANNs consisted of three layers: an input layer (Input), one hidden layer and an output layer (Output). To train the data, the Broyden-Fletcher-Goldfarb-Shanno algorithm (BFGS) was used. An automated search for regression neural networks, either multilayer perceptron networks (MLP) or radial basis function networks (RBF), was conducted and trained a total of 100 networks, and the best network was selected based on their performances during training, testing and validation process. During training, the following activation functions for hidden and output neurons were tested: identity, logistic, Tanh, exponential and sine.

The dataset was divided into a training set ($n = 25$) and a test set ($n = 9$) (Table 1). The training dataset was used for model building, and the test dataset was used to validate the models. The test set was selected to cover all structure types and the $\log P_c$ range used. The test set was further divided into a verification set ($n = 4$) and a test set ($n = 5$) for modeling ANN.

Model validation

In general, the internal validation of the model can be performed with a couple of statistical parameters, such as the coefficient of determination for the training set (R^2), the cross-validated correlation coefficient (Q^2) and the root mean square error of estimation (RMSEE) [31]. Tenfold cross-validation technique was used to calculate the Q^2 parameter. The models were also subjected to external validation analysis [31], which evaluates the reliability of the models by calculating the coefficient of determination for the test set (R^2_{pred}) and the root mean square error of prediction (RMSEP). For further evaluation of the predictive abilities, the r -metrics [32] parameters (r^2_m , r^2_{m})

and Δr_m^2) were calculated. The Concordance correlation coefficient (CCC) [33] has also been calculated to check the model reliability.

For the QSPR model to be applied accurately, it is essential to establish its applicability domain (AD) [34]. The AD is a theoretical region in chemical space determined by the structures of compounds in the training set [35]. SPSS v.18.0 software [36] was used to perform the leverage approach, which determines if a structure's activity can be reliably predicted by comparing its leverage value h to the critical value h^* . The critical value can be calculated using the following Equation 3:

$$h^* = \frac{3(p+1)}{n} \quad (\text{Equation 3})$$

In which p represents the number of model variables while n represents the number of structures used to create the model.

Results & discussion

The main objective of the current study was to determine the effective BBB permeability of the 34 protein kinase inhibitors using the PAMPA-BBB assay and perform QSPR analysis to establish the most reliable *in silico* models for permeability prediction. The created QSPR model with the highest predictive capacity was used for mechanistic interpretation of the structural factors important for passive diffusion of these and similar compounds through the BBB and provide a robust model for permeability prediction of structurally similar derivatives.

Parallel artificial membrane permeability assay results

The PAMPA-BBB assay was performed to provide information on the passive diffusion rate of the FDA-approved kinase inhibitors through the BBB and estimate the potential passage rate of the experimental compounds. The test has also been performed for levodopa and haloperidol with and without the addition of Tween-80. Levodopa is a hydrophilic compound known to utilize active transport and does not passively diffuse through BBB [37]. On the other hand, haloperidol is a highly lipophilic compound, with a strong affinity for lipids and can readily cross cell membranes, including the BBB [38]. Since Levodopa does not pass into the acceptor wells and haloperidol passes in almost the same amount in both cases, it has been confirmed that the Tween-80 does not affect the viability of the PAMPA testing in our work.

By using calculated experimental permeability rates ($\log P_e$), compounds can generally be classified into four groups: impermeable compounds ($\log P_e < -6.14$), low permeability compounds ($-6.14 < \log P_e < -5.66$), medium permeability compounds ($-5.66 < \log P_e < -5.33$) and high permeability compounds ($\log P_e > -5.33$) [39]. Obtained results showed that the examined kinase inhibitors vary between low, medium and high permeable compounds, as presented in Table 2.

Performed hierarchical clustering showed that most clusters have representative structures in the dataset, implying that the compounds used in the study cover a broad range of structural features of existing kinase inhibitors.

By comparing the clinical data with our results, considering the structural characteristics of these compounds, some agreement can be observed (Table 2).

The obtained $\log P_e$ values for two BCR-ABC tyrosine kinase inhibitors indicated medium and high BBB permeability of nilotinib and imatinib respectively. Due to its higher permeability and lower adverse effects compared with nilotinib, imatinib could be a potential candidate for the treatment of CNS tumors. On the other hand, lower permeability of nilotinib could be described by the presence of a bulky trifluoromethyl substituent. Among the EGFR/HER kinase inhibitors, lapatinib showed high permeability, which could be associated with the lipophilic substituent on the 4-anilinoquinazoline moiety. Nintedanib, as a VEGFR inhibitor, was classified as low permeable, which could be due to several structural features, such as the bulky substituent at the alpha position of the 1H-indolinone and its four amino groups, which could be protonated at physiological pH (7.4).

Everolimus, for example, is an agent known to be able to cross the BBB [40] and results obtained herein also showed that it can be classified as a highly permeable agent. In addition, high permeability in PAMPA assay was proposed for fasudil that has been shown to have a good therapeutic effect on neurodegenerative diseases, as it increases the activity of neural stem cells [41]. Moreover, fasudil was reported to reduce BBB dysfunction, improve neurological and clinical outcomes in acute cerebral infarction and have neuroprotective effects [42].

Table 2. The parallel artificial membrane permeability assay-blood–brain barrier results for the studied kinase inhibitors.

Compound	P_e	$\log P_e$
Compound 2	1.13E-06	-5.95
Vemurafenib	1.17E-06	-5.93
Afatinib	1.47E-06	-5.83
Nintedanib	1.60E-06	-5.80
Compound 1 [†]	1.57E-06	-5.80
Compound 5	1.63E-06	-5.79
Compound 16	1.63E-06	-5.79
Compound 8 [†]	1.76E-06	-5.75
Compound 7	1.99E-06	-5.70
Compound 14	2.08E-06	-5.68
Compound 17	2.29E-06	-5.64
Gefitinib [†]	2.37E-06	-5.62
Nilotinib	2.38E-06	-5.62
Compound 10 [†]	2.47E-06	-5.61
Compound 11	2.52E-06	-5.60
Compound 13 [†]	2.57E-06	-5.59
Compound 9	2.85E-06	-5.55
Compound 4	2.94E-06	-5.53
Compound 6	3.12E-06	-5.51
Compound 3 [†]	3.24E-06	-5.49
Crizotinib	3.50E-06	-5.46
RKI-1447 [†]	3.74E-06	-5.43
Erlotinib	3.79E-06	-5.42
Compound 12	3.85E-06	-5.41
Imatinib	4.73E-06	-5.32
Dabrafenib	4.88E-06	-5.31
Lapatinib	4.85E-06	-5.31
Compound 15	5.31E-06	-5.28
N-desmethylinatinib [†]	5.40E-06	-5.27
Fasudil	6.72E-06	-5.17
Everolimus	7.52E-06	-5.12
Ruxolitinib [†]	7.59E-06	-5.12
Axitinib	1.19E-05	-4.93
Palbociclib	2.07E-05	-4.68

[†] Test set compounds.

Nevertheless, crizotinib does not pass the BBB, but our results showed that it has moderate passive permeability. However, the reason that crizotinib cannot pass the BBB is that it is a known P-gp substrate [43], which could be the explanation for this discrepancy.

The PAMPA-BBB results for the experimental compounds showed that aryl-2-aminopyridines (compounds 1 and 2) were classified as low-permeable compounds, and pyridyl-based thiazolyl-hydrazones and selenazolyl-hydrazones (compounds 3–17) fall into the low-, medium- and high-permeability categories.

Furthermore, the ADMET predictor was utilized to predict whether examined compounds are P-glycoprotein (Pgp) substrates or not (Supplementary Table 2). Dabrafenib, erlotinib, fasudil, lapatinib, nilotinib and ruxolitinib, as well as all experimental compounds were predicted not to be Pgp substrates. The remaining portion of the dataset compounds were expected to act as both Pgp substrates and inhibitors. The only exception was RKI-1447 which was predicted only to be a Pgp substrate. Plasma protein binding was also predicted with the ADMET predictor to estimate the percentage of the drug in plasma available to be subjected for passive diffusion across the BBB. All compounds except fasudil, palbociclib and ruxolitinib had less than 10%, but more than 1% unbound drug. Despite the belief that drugs bound to plasma proteins are not available for transport via the BBB, studies have

Table 3. Results of the validation parameters for created quantitative structure–property relationship models.

	R^2	Q^2	RMSEE	R^2_{pred}	RMSEP	r^2_m	$r^2_{/m}$	Δr^2_m	$(r^2_m + r^2_{/m})/2$	$(r^2_m - r^2_{/m})/r^2_m$	k'	CCC
MLR	0.769	0.628	0.145	0.811	0.092	0.791	0.676	0.115	0.734	0.036	0.997	0.897
SVM	0.839	0.648	0.121	0.777	0.100	0.723	0.657	0.067	0.690	0.033	1.002	0.882
ANN	0.749	0.620	0.151	0.953	0.050	0.978	0.988	0.010	0.983	0.000	0.992	0.977
				0.999 [†]	0.095 [†]							
Limit	>0.7	>0.5		>0.5		>0.5	>0.5	<0.2	>0.5	<0.1	0.85 < k' < 1.15	>0.85

[†]Verification set validation parameters.
 ANN: Artificial neural network; CCC: Concordance correlation coefficient; MLR: Multiple linear regression; RMSEE: Root mean square error of estimation; RMSEP: Root mean square error of prediction; SVM: Support vector machine regression.

demonstrated that numerous plasma protein bound compounds are able to pass the BBB. This would mean that drug concentration in the brain can be achieved by intrinsic passive permeability of the compounds and that the PAMPA test is a viable tool for early passive BBB permeability screening [44].

Quantitative structure–property relationship modeling

QSPR modeling was performed with the aim to investigate possible correlations between molecular properties of the examined PK inhibitors and related compounds and BBB permeability. The preprocessed descriptors obtained for the dataset compounds were used as independent variables, and the calculated $\log P_e$ value from PAMPA assay was selected as the dependent variable. Stepwise MLR modeling was applied to estimate the linear relationship between the descriptors and the $\log P_e$ value and to find the most important variables that could efficiently predict and describe passive permeability through BBB. After stepwise selection, four variables were selected by the MLR model based on F to enter and F to remove criteria. The model was subjected to both internal and external validation, including r -metrics.

The radial basis function (RBF) was used as the kernel for modeling SVM. To optimize the parameters C , ϵ and γ , their values were changed in the range of 10–45 for C , 0.01–0.10 for ϵ , and 0.10–1.00 for γ . After hyperparameter optimization, the optimal values for C , ϵ , and γ were 10, 0.1 and 0.25, respectively.

A three-layer MLP neural network, with the final structure of 4-8-1, was selected as ANN with the highest training performance. The activation function of the hidden neurons was sinusoidal, and the activation function of the output neurons was logistic.

Comprehensive internal and external statistical validation analysis revealed that all created QSPR model possess comparable performances (Table 3). However, a slight advantage may be given to the QSPR-SVM model (Figure 1) which satisfied all critical validation parameters, making it an effective tool for predicting the permeability of new analogs within the defined applicability domain. The ANN model has higher external parameters probably due to the smaller number of test set compounds.

SVM models are useful in avoiding over-fitting problems while providing accurate results with a small amount of data. Although ANN have the advantage of fitting datasets well, it can sometimes hinder their performance. Additionally, unlike SVM, ANN tends to overfit models, thereby reducing its generalization ability.

Created MLR, ANN and SVM models were analyzed to determine their applicability domain using chosen molecular descriptors. The leverage value limit was calculated using Eq. 10 and found to be $h^* = 0.6$. The Williams plot shown in Figure 2 indicated that both the training and test sets compounds were within the defined AD. Thus, the SVM model created is suitable for predicting the BBB permeability of new compounds with similar structures. This could be particularly useful in optimizing novel ligands to develop more effective and potent compounds targeting CNS disorders or to prevent potential adverse effects on the CNS.

Quantitative structure–property relationship models interpretation

Molecular descriptors play an essential role in QSPR modeling, providing chemical-structural information of the molecule's structure in numeric form. In that regard, reliable prediction of the molecular descriptors is a crucial step for developing predictable QSPR models. The created and validated MLR model was assessed to elucidate the encoded structure–permeability relationships, identifying four variables that significantly describe the $\log P_e$ value. The selected parameters and coefficients are listed in Table 4.

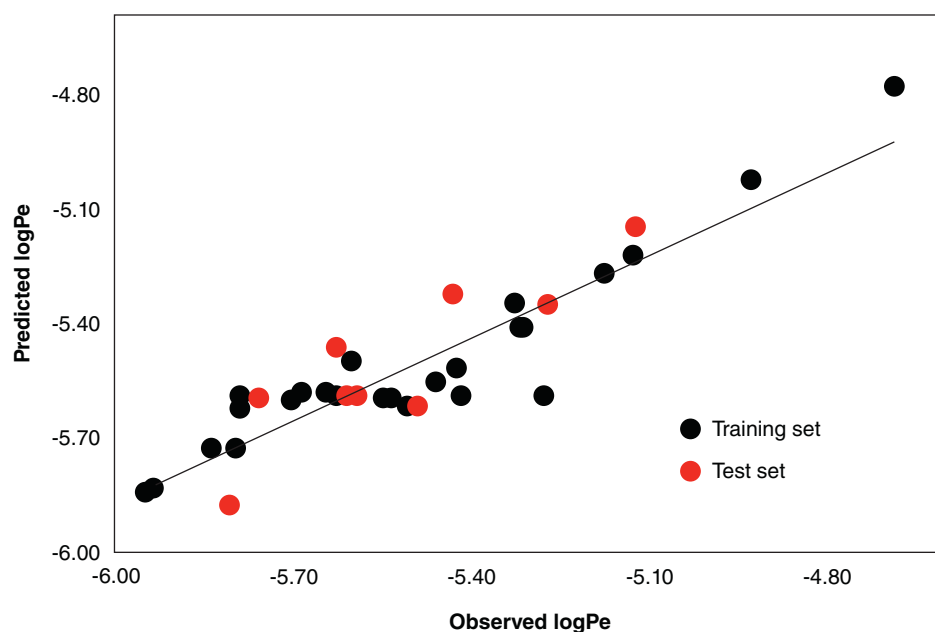


Figure 1. The plot of the dataset's observed versus predicted $\log P_e$ values for the quantitative structure–property relationship–support vector machine regression model.

- – Training set.
- – Test set.

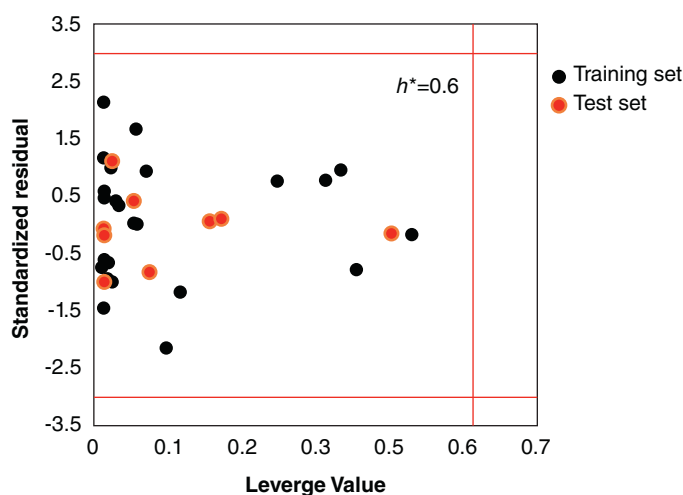


Figure 2. The applicability domain of the created quantitative structure–property relationship–support vector machine regression model.

- – Training set.
- – Test set.

Table 4. The most influential molecular descriptors from quantitative structure–property relationship study.

Descriptors	CATS2D_09_DA	CATS2D_04_AA	B04[N-S]	F07[C-N]	Intercept
Coefficients	-0.121	0.171	0.170	0.096	-5.496

The MLR coefficients of normalized parameters indicated that CATS2D_04_AA was the highest positive contributor to the $\log P_e$ value (Supplementary Figure 3). Furthermore, B04[N-S] and F07[C-N] were also identified to positively correlate with passive permeability through BBB, while CATS2D_09_DA was observed to have negative impact.

ANN and SVM regression models were developed to investigate possible nonlinear relationships between selected descriptors and $\log P_e$ value. After sensitivity analysis, the created ANN model showed similar contributions to the $\log P_e$ value compared with the MLR model. CATS2D_09_DA was the most influential descriptor on the permeation coefficient through the PAMPA artificial membrane.

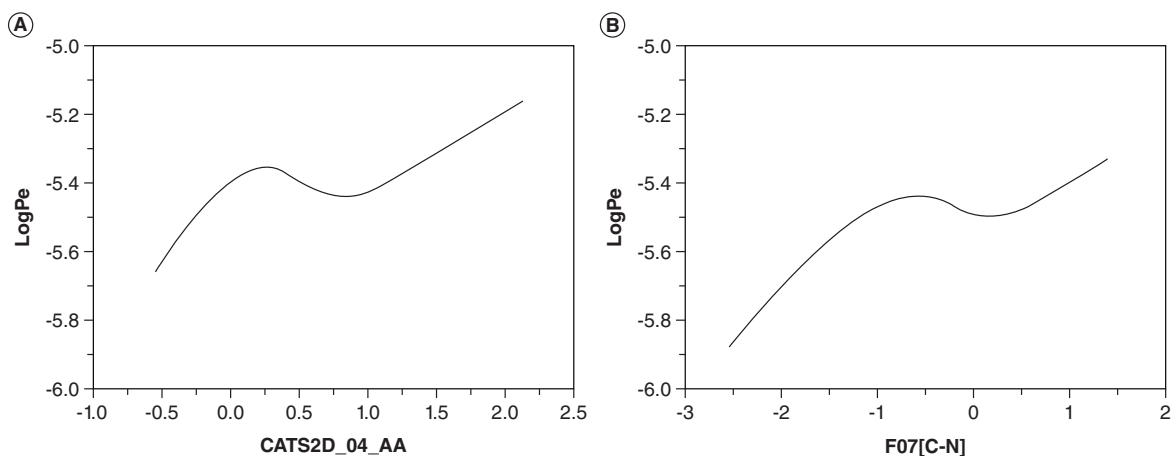


Figure 3. The response graphs of selected descriptors from the quantitative structure–property relationship-support vector machine regression model. (A) CATS2D_04_AA and (B) F07[C-N].

The response graphs (Figure 3) of CATS2D_04_AA and F07[C-N] show a nonlinear dependence on $\log P_e$ in QSPR-SVM modeling. The more precise relationship of these descriptors to the dependent variable is the likely reason for the higher performance of this QSPR model.

One of the descriptors with a positive contribution to the $\log P_e$ value is CATS2D_04_AA, which belongs to the CATS2D descriptors describing a distance between different pharmacophoric features [45]. It is defined as CATS2D acceptor-acceptor at lag (topological distance) of 4. Most of the studied compounds, which had lower permeability, had a descriptor value of 0. Compounds with higher permeability had higher values, such as palbociclib and ruxolitinib. On the other hand, the descriptor coefficient of CATS2D_09_DA had a negative value, which means that the hydrogen bond donor and acceptor with the topological distance of 9 lowers the $\log P_e$ value. This implies that the presence of hydrogen bond donors in this group of compounds would decrease the penetration through the BBB. Vemurafenib is one of compounds from the dataset that had the descriptor value of 2 and a lower $\log P_e$ value.

The atom-pair fragment descriptor B04[N-S] [45] defines the frequency of pairs of N and S atoms with a topological distance of 4, indicating that sulfur-containing compounds should have an optimal distance between nitrogen and sulfur in their structure. Axitinib is one of the compounds with high permeability that exhibited this property. Another pair of C and N atoms with a topological distance of 7 also positively contributed to the $\log P_e$ value, defined by the descriptor F07[C-N]. A larger distance between nitrogen and a carbon atom described by this descriptor could indicate that the presence of carbonaceous side chains connected to central aromatic rings would enhance penetration through the BBB.

Described descriptors provide valuable insight into possible ways to optimize BBB penetration of the kinase inhibitors and structurally related studied compounds. Structural modifications, such as reducing the number of hydrogen bond donors, for example, by replacing free hydrogens with alkyl chains, or isosteric replacement with some hydrogen bond acceptors, such as nitrogen, at the parts of the structure insignificant for activity or selectivity, may prove to be promising strategies to improve the BBB penetration of these structures. For instance, if we add an N-methyl group to the sulfonamide part of vemurafenib (compound **V1**), the compound with the second $\log P_e$ value in our dataset, it will decrease the number of hydrogen bond donors in the structure, while keeping the 7-azaindole ring intact, which is responsible for binding with the BRAF hinge region. This modification would result in increased passive permeability across the BBB. Based on the PAMPA-SVM model, the predicted $\log P_e$ value of the compound **V1** is -5.35, which falls within the upper limit of medium permeable compounds.

When comparing our model to existing models in the literature, various authors have used a QSPR approach to uncover the significant impact of different descriptors on BBB permeability. Zhang *et al.* [46] addressed the importance of E-state and Van der Waals surface areas in addition to traditional factors like lipophilicity, hydrogen-bonding properties and polar surface area on BBB permeability. Barn *et al.* [47] have highlighted the influence of polar surface area, molecular weight, lipophilicity, and hydrogen bond donors. Wager *et al.* [48] also described the influence of lipophilicity factors, molecular weight, topological polar surface area, presence of hydrogen bond

donors and pKa values on blood–brain permeability. Our model aligns with these findings regarding the primary descriptors influencing BBB permeability while also differentiating itself through specific structural features essential for designing and optimizing kinase inhibitors.

Conclusion

In this study, the PAMPA *in vitro* technique was used to predict the ability of structurally different kinase inhibitors and related compounds to penetrate the BBB. The experimentally determined permeability values ($\log P_e$) were calculated and the tested compounds can be characterized as low, medium and high permeable compounds. The $\log P_e$ values and calculated molecular descriptors were used for development of different models which can reliably predict BBB permeability. To evaluate the reliability of the models, they were subjected to extensive statistical validation and the PAMPA-SVM model has been selected for further analysis. It was found that the descriptors CATS2D_09_DA, CATS2D_04_AA, B04[N-S] and F07[C-N] have the greatest influence on the $\log P_e$ values. The specified descriptors provide important information for optimizing BBB penetration of kinase inhibitors and structurally related compounds. One approach is to reduce hydrogen bond donors. The integration of the PAMPA technique with *in silico* computational methods offers a reliable and cost-effective approach for assessing penetration of kinase inhibitor candidates targeting the central nervous system, crucial in pharmaceutical research for optimizing drug delivery and developing effective treatments through the BBB.

Summary points

- The parallel artificial membrane permeability assay *in vitro* technique was used to predict the ability of diverse kinase inhibitors and related compounds to penetrate the blood–brain barrier (BBB).
- The experimentally determined permeability values ($\log P_e$) and the calculated molecular descriptors were analyzed using three different statistical and mathematical methods - multiple linear regression (MLR), artificial neural network (ANN) and support vector machine (SVM) to develop linear and nonlinear quantitative structure–property relationship (QSPR) models which can reliably predict BBB permeability.
- To evaluate the predictive ability of the models, they were subjected to extensive statistical validation. The results showed that the SVM model has good predictive power based on the validation parameters, making it an effective tool for predicting the permeability of new analogs within the defined applicability domain.
- It was found that the descriptors CATS2D_09_DA, CATS2D_04_AA, B04[N-S], and F07[C-N] have the greatest influence on the $\log P_e$ values. The integration of this technique with *in silico* computational methods provided a reliable, rapid and cost-effective methodology for assessing brain penetration.
- The structural diversity of the compounds in the dataset and good predictive power of the created model indicated the potential utility of these methods for assessing permeability through the BBB of related kinase inhibitor candidates targeting CNS.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/fmc-2023-0390

Author contributions

M Jovanović: conceptualization, methodology, software, writing – original draft. M Radan: methodology, supervision, writing – review & editing. M Čarapić: formal analysis, writing – original draft. N Filipović: writing – review & editing. K Nikolic: supervision, conceptualization, methodology, resources, writing – review & editing. M Crevar: supervision, conceptualization, methodology, formal analysis, writing – review & editing.

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Competing interests disclosures

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

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УНИВЕРЗИТЕТ У БЕОГРАДУ
ФАРМАЦЕУТСКИ ФАКУЛТЕТ

Посл.број: 3/128

Дана 07.11.2025. године

На основу Статута Универзитета у Београду, продекан за последипломску наставу и континуирану едукацију Факултета је дана 07.11.2025. године, донела

ОДЛУКУ

ОДОБРАВА се МИЛАНУ ЈОВАНОВИЋУ, студенту Фармацеутског факултета, индекс бр. 11/2018, **продужетак рока за завршетак студија у школској 2025/2026 години.**

Образложење

МИЛАН ЈОВАНОВИЋ, студент на студијском програму ДАС – Фармацеутска хемија, поднео је захтев заведен под пословним бројем 3/128 од 05.11.2025. године продекану за последипломску наставу и континуирану едукацију Факултета да му се одобри **продужетак рока за завршетак студија у школској 2025/2026 години.**

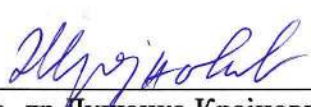
Продекан а последипломску наставу и континуирану едукацију Факултета је разматрала захтев 07.11.2025.године и оценила да је захтев основан, те је донето решење као у диспозитиву.

ПРАВНА ПОУКА: Против ове одлуке именовани има право приговора декану Факултета у року од 8 (осам) дана од дана пријема истог.

Одлуку доставити: студенту, Одсеку за наставу и студентска питања.

ПРОДЕКАН ЗА ПОСЛЕДИПЛОМСКУ
НАСТАВУ
И КОНТИНУИРАНУ ЕДУКАЦИЈУ




Проф. др Душанка Крајновић

УНИВЕРЗИТЕТ У БЕОГРАДУ
ФАРМАЦЕУТСКИ ФАКУЛТЕТ
Посл.број: 5/133
Дана 24.09.2024.године

На основу члана 107. Закона о високом образовању Републике Србије, продекан Факултета је дана 24.09.2024. године, донела

РЕШЕЊЕ

ОДОБРАВА се ЈОВАНОВИЋ МИЛАНУ, студенту Фармацеутског факултета, индекс бр. 11/2018, продужетак рока за завршетак студија у школској 2024/2025.

Образложење

ЈОВАНОВИЋ МИЛАН, студент на студијском програму ДАС – Фармацеутска хемија, поднео је захтев бр. 5/133 од 24.09.2024. године продекану за последипломске студије Факултета да му се одобри **продужетак рока за завршетак студија у школској 2024/2025.**

Продекан Факултета је разматрала захтев 24.09.2024. године и оценила да је захтев основан, те је донето решење као у диспозитиву.

ПРАВНА ПОУКА: Против овог решења именовани има право приговора декану Факултета у року од 8 (осам) дана од дана пријема истог.

Решење доставити: Именованом, декану, продекану за наставу, секретару, Одсеку за наставу и студентска питања и архиви.

ПРОДЕКАН ЗА ПОСЛЕДИПЛОМСКУ НАСТАВУ
И КОНТИНУИРАНУ ЕДУКАЦИЈУ




Проф. др Сандра Везмар Ковачевић