Chapter 7

Appendices

7.1 Summary

This thesis describes the application and evaluation of rational drug design methods for predicting and interpreting the light-dependent activity of photopharmacogical agents. In **Chapter 1** we analyzed the state of the art of informed design in photopharmacology, with a focus on *E*/*Z* molecular photoswitches and computer-aided strategies. In most cases, photoswitchable ligands have been designed as analogs of existing compounds. Starting from a known bioactive structure, photoswitches can be attached to or inserted into it, can replace a bioisosteric substructure, or they can serve as a spacer. Furthermore, azobenzene-containing drugs can be repurposed for photopharmacology or their light-induced changes in potency can be inverted by introducing diazocines in place of azobenzene. However, we identified several underexplored design approaches. These include the use of Lipinski's rule of five, comparisons of 3D structures and electronic properties between bioisosteres and specific photoisomers, and fragment-based drug discovery.

The implementation of molecular docking and molecular dynamics (MD) simulations was reported in **Chapter 2** for different photopharmacological studies. Docking calculations supported the introduction of photocleavable protecting groups to mask carboxylic acid moieties in a MDM2 inhibitor and a probe for cGAS enzyme. Moreover, docking studies on photoswitchable $BRAF^{V600E}$ kinase inhibitors revealed striking similarities in the binding conformations of biaryl sulfonamides and (*Z*)-azobenzene, which were further investigated in **Chapter 4**. Finally, conformational searches and MD simulations guided the design of amino acid linkers for light-controlled polyglutamate aggregation, as well as helped the interpretation of the behavior of azobenzene-based DNA glues that showed chirality transfer.

Chapter 3 introduced the adaptation of the drug discovery concept of design-make-test-analyze (DMTA) cycles into design-make-switch-test-analyze (DMSTA) cycles for photopharmacology. By taking *Escherichia coli* dihydrofolate reductase (eDHFR), an important target for antibiotic research, as a model case, we explored several structure-based hypotheses for the informed design of (*Z*)-active photoswitchable antibiotics. For the identification of a photoswitchable hit, the incorporation of a photoresponsive unit in the pharmacophore of trimethoprim did not result in a (*Z*)-active inhibitor despite the expected formation of the key hydrogen-bond network only in the (*Z*)-state. However, azologization of the *meta*-substituted biaryl scaffold of propargyl-linked trimethoprim analogs generated the desired (*Z*)-active hit. Subsequent attempts at enhancing the photostationary distribution of the irradiated samples did not increase the differences in potency of the eDHFR inhibitors before and after irradiation. On the other hand, targeting a hydrophobic subpocket of the enzyme and a specific salt bridge only with the metastable (*Z*)-form emerged as the most promising design strategies. This systematic evaluation of rational drug design approaches led to the identification of three light-activated eDHFR inhibitors with potencies in the low-nanomolar range.

Starting from the azologization strategy, **Chapter 4** extended its scope by defining the concept of (*Z*)-like azosteres for the design of photoswitchable drugs that are more active

in their metastable (*Z*)-state. Generic biaromatic systems linked by two atoms were compared to (*Z*)-azobenzene in terms of structural and electronic features, such as centroid angles, ring distances, electrostatic potential (ESP) surfaces and dipole moments. Biaryl sulfonamides were identified as promising (Z)-like azosteres because of their centroid angle < 100° and ring distance < 5 Å, as well as favorable ESP surface and dipole moment of 5.8 D, which is better approximated by (*Z*)-azobenzene (3.0 D) than (*E*)-azobenzene (0 D). The azologization of biaryl sulfonamides was explored for the design of HDAC2 and Lp-PLA_2 inhibitors. The pharmacophoric role of the substructure in the parent ligand had a clear influence on the outcome of the azologization. Since the -SO_2NH_2- linker engaged in hydrogen bonding interactions with buried residues of Lp-PLA_2, its azolog suffered a larger loss of activity compared to the parent inhibitor. At the same time, the irradiated sample showed a > 10-fold increase in potency compared to the thermally adapted sample, indicating that a (*Z*)-like substructure promoted biological activity. On the other hand, when the biaryl sulfonamide served as a solvent-exposed moiety to ensure optimal hydrophobic contacts of belinostat with HDAC2, its azolog maintained the original potency and displayed a smaller, 2-fold difference in activity before and after irradiation.

In **Chapter 5**, we expanded the notion of (*Z*)-like azosteres to one-atom-linked biaryl systems. Regarding molecular geometry, benzophenone emerged as a promising (*Z*)-like substructure because of its suitable ring angle (> 40°) and ring distance (~ 5 Å). Additionally, the experimental dipole moments of benzophenone (3.0 D) and (*Z*)-azobenzene (3.0 D) are identical, whereas (*E*)-azobenzene has a null dipole moment. With these data in hand, we tested the scope of the azologization of benzophenones by designing photoswitchable analogs of a circadian clock regulator and of ketoprofen, a COX-2 inhibitor. While we proved the validity of our approach by achieving reversible regulation of the circadian period with visible light in the first example, we only obtained unconclusive results with our light-controlled COX-2 inhibitors because of interference with the biological assay.

Finally, **Chapter 6** explored the use of a MD-based method for the interpretation of the differences in potency between the photoisomers of azobenzene-containing inhibitors. Photopharmacology would greatly benefit from quantitative methods for estimating the binding affinities of photoswitchable ligands. We employed umbrella sampling (US) simulations to calculate the binding affinities of three photoswitchable inhibitors, targeting HDAH, HDAC2 and Lp-PLA_2. In all cases, the calculated ΔGs of binding did not replicate the experimental differences in biological activity. Further studies need to improve the correlation with published data by using longer simulations to relax the protein-ligand complex more thoroughly and to ensure convergence of the US windows.

In conclusion, the work presented in this thesis represents a significant step toward rational drug design in photopharmacology. A wide range of informed, computer-aided design strategies was applied to the development of light-controlled tools, spanning from antibiotics to chemotherapeutics. The employed computational methods included docking and MD simulations for structure-based design, database searches to investigate molecular geometries, DFT calculations to study electronic properties. Future efforts in

rational photopharmacology will need a combination of different methodologies and approaches to shed more light on the optical control of bioactive compounds.

7.2 Samenvatting

Dit proefschrift beschrijft de toepassing en evaluatie van rationele methoden voor het ontwerpen van geneesmiddelen voor het voorspellen en interpreteren van de lichtafhankelijke activiteit van fotofarmacologische middelen. In **Hoofdstuk 1** analyseerden we de stand van zaken van geïnformeerd ontwerp in fotofarmacologie, met een focus op *E/Z* moleculaire fotoschakelaars en computerondersteunde strategieën. In de meeste gevallen zijn de fotoschakelbare liganden ontworpen als analogen van bestaande verbindingen. Uitgaande van een bekende bioactieve structuur kunnen fotoschakelaars eraan worden bevestigd of erin worden geplaats, een bio-isosterische onderbouw vervangen of als spacer dienen. Bovendien kunnen azobenzeen bevattende geneesmiddelen worden hergebruikt voor fotofarmacologie. Ook kan hun door licht geïnduceerde veranderingen in potentie kunnen worden omgekeerd door in plaats van azobenzeen, diazocines te introduceren. Desalniettemin hebben we hebben verschillende onderbelichte ontwerpbenaderingen geïdentificeerd. Deze omvatten het gebruik van Lipinski's regel van vijf, vergelijkingen van 3D-structuren en elektronische eigenschappen tussen bio-isosteren en specifieke foto-isomeren, en op fragmenten gebaseerde medicijnontdekking.

De implementatie van moleculaire docking en moleculaire dynamica (MD) simulaties is beschreven in **Hoofdstuk 2** voor verschillende fotofarmacologische studies. Docking-berekeningen ondersteunden de introductie van fotobeschermgroepen om carbonzuren te maskeren in een MDM2-remmer en een probe voor cGAS-enzym. Bovendien onthulde de docking-studies met fotoschakelbare $BRAF^{V600E}$-kinaseremmers opvallende overeenkomsten in de bindingsconformaties van biarylsulfonamiden en (*Z*)-azobenzeen, die verder werden onderzocht in **Hoofdstuk 4**. Ten slotte leidden conformationele zoekopdrachten en MD-simulaties het ontwerp van aminozuurlinkers voor licht-gecontroleerde polyglutamaataggregatie, en hielp het interpretatie van het gedrag van op azobenzeen gebaseerde DNA-lijmen die chiraliteitsoverdracht vertoonden.

Hoofdstuk 3 introduceerde de aanpassing van het drug discovery concept van design-make-test-analyse (DMTA) rondes in design-make-switch-test-analyse (DMSTA) rondes voor fotofarmacologie. Door *Escherichia coli* dihydrofolaatreductase (eDHFR), een belangrijk doelwit voor antibioticaonderzoek, als een modelcase te nemen, hebben we verschillende op structuur gebaseerde hypothesen onderzocht voor het geïnformeerde ontwerp van (*Z*)-actieve fotoschakelbare antibiotica. Voor de identificatie van een fotoschakelbare kandidaat resulteerde de opname van een fotoresponsieve eenheid in de farmacofoor van trimethoprim niet in een (*Z*)-actieve remmer, ondanks de verwachte vorming van het belangrijkste waterstofbrugnetwerk dat alleen voorkomt in de (*Z*)-toestand. Azologisatie van de meta-gesubstitueerde biaryl-scaffold van propargyl-gekoppelde trimethoprim-analogen genereerde echter de gewenste (*Z*)-actieve hit. Daaropvolgende pogingen om de fotostationaire verdeling van de bestraalde monsters te

verbeteren, hebben de verschillen in potentie van de eDHFR-remmers voor en na bestraling niet vergroot. Daarentegen kwam het richten op een hydrofobe subpocket van het enzym en een specifieke zoutbrug met alleen de metastabiele (*Z*)-vorm naar voren als de meest veelbelovende ontwerpstrategie. Deze systematische evaluatie van rationele benaderingen voor het ontwerpen van geneesmiddelen leidde tot de identificatie van drie licht geactiveerde eDHFR-remmers met potenties in het lage nanomolaire bereik.

Beginnend met de azologiseringsstrategie, breidde **Hoofdstuk 4** zijn bereik uit door het concept van (*Z*)-achtige azosteren te definiëren voor het ontwerp van fotoschakelbare geneesmiddelen die actiever zijn in hun metastabiele (*Z*)-staat. Algemene twee-atoom-gekoppelde biarylsystemen werden vergeleken met (*Z*)-azobenzeen in termen van structurele en elektronische kenmerken, zoals zwaartepunthoeken, ringafstanden, elektrostatische potentiaal (ESP) oppervlakken en dipoolmomenten. Biarylsulfonamiden werden geïdentificeerd als veelbelovende (*Z*)-achtige azosteren vanwege hun zwaartepunthoek < 100° en ringafstand < 5 Å, evenals gunstig ESP-oppervlak en dipoolmoment van 5.8 D, wat beter wordt benaderd door (*Z*)-azobenzeen (3.0 D) dan (*E*)-azobenzeen (0 D). De azologisering van biarylsulfonamiden werd onderzocht voor het ontwerp van HDAC2- en Lp-PLA2-remmers. De farmacofore rol van de substructuur in het moederligand had een duidelijke invloed op de uitkomst van de azologisering. Omdat de -SO_2NH_2-linker betrokken was bij waterstofbindingsinteracties met niet-oppervlakkige residuen van Lp-PLA_2, gaf zijn azolog een groter verlies aan activiteit in vergelijking met de oorspronkelijke remmer. Tegelijkertijd vertoonde het bestraalde monster een > 10-voudige toename in potentie in vergelijking met het thermisch aangepaste monster, wat aangeeft dat een (*Z*)-achtige substructuur de biologische activiteit bevorderde. Echter, wanneer de biarylsulfonamide diende als een aan oplosmiddel blootgestelde groep om optimale hydrofobe contacten van belinostat met HDAC2 te verzekeren, behield zijn azolog de oorspronkelijke potentie en vertoonde een kleiner, 2-voudig, verschil in activiteit voor en na bestraling met licht.

In **Hoofdstuk 5** hebben we de notie van (*Z*)-achtige azosteren uitgebreid tot één-atoom-gekoppelde biarylsystemen. Wat betreft de moleculaire geometrie, kwam benzofenon naar voren als een veelbelovende (*Z*)-achtige substructuur vanwege de geschikte ringhoek (> 40°) en ring afstand (~ 5 Å). Bovendien zijn de experimentele dipoolmomenten van benzofenon (3.0 D) en (*Z*)-azobenzeen (3.0 D) identiek, terwijl (*E*)-azobenzeen een dipoolmoment van nul heeft. Aan de hand van gegevens hebben we het bereik van de azologisering van benzofenonen getest door fotoschakelbare analogen te ontwerpen van een circadiane klokregulator en van ketoprofen, een COX-2-remmer. Hoewel we de validiteit van onze aanpak hebben bewezen door in het eerste voorbeeld omkeerbare regulatie van de circadiane periode met zichtbaar licht te bereiken, hebben we helaas niet-overtuigende resultaten verkregen met onze lichtgestuurde COX-2-remmers vanwege interferentie met de biologische test.

Ten slotte onderzochten we in **Hoofdstuk 6** het gebruik van een MD-gebaseerde methode voor de interpretatie van de verschillen in potentie tussen de foto-isomeren van azobenzeenbevattende remmers. Fotofarmacologie zou veel baat hebben bij kwantitatieve methoden voor het schatten van de bindingsaffiniteiten van fotoschakelbare

liganden. We hebben simulaties van umbrella sampling (US) gebruikt om de bindingsaffiniteiten van drie fotoschakelbare remmers te berekenen, gericht op HDAH, HDAC2 en Lp-PLA$_2$. In alle gevallen repliceerden de berekende ΔG's van binding niet de experimentele verschillen in biologische activiteit. Verdere studies moeten de correlatie met gepubliceerde gegevens verbeteren door langere simulaties te gebruiken om het eiwit-ligandcomplex grondiger te ontspannen en om convergentie van de US vensters te verzekeren.

Concluderend, het werk dat in dit proefschrift wordt gepresenteerd, vertegenwoordigt een belangrijke stap in de richting van rationeel medicijnontwerp in de fotofarmacologie. Een breed scala aan geïnformeerde, computerondersteunde ontwerpstrategieën werden toegepast bij de ontwikkeling van lichtgecontroleerde bioactieve moleculen, variërend van antibiotica tot chemotherapeutica. De gebruikte computationele methoden omvatten docking- en MD-simulaties voor structuurgebaseerd ontwerp, databasezoekopdrachten om moleculaire geometrieën te onderzoeken, DFT-berekeningen om elektronische eigenschappen te bestuderen. Toekomstige inspanningen in rationele fotofarmacologie zullen een combinatie van verschillende methodologieën en benaderingen nodig hebben om meer licht te werpen op de optische controle van bioactieve verbindingen.

7.3 Popular summary

Modern medicine has improved our quality of life and reduced human suffering through the discovery of countless drugs. Whenever you take a pill, its active ingredient acts as a key for a specific lock in your body (the biological target) that is responsible for or linked to your sickness. More accurately, the target also adapts itself to the incoming drug, the same way as a glove accommodates a hand. Once the lock is blocked with the key, this triggers a biological effect that reduces the symptoms or cures the disease.

Unfortunately, drugs also cause undesired side effects, as the keys interact with locks in parts of the body that are different from the therapeutic site. The emerging field of photopharmacology tries to solve such behavior by designing drugs that can be locally activated with light. In a nutshell, we modify the original molecule with a molecular photoswitch, which can be toggled between “on” and “off” states by irradiation with light. Thanks to this switch, we can control the 3D shape and the electronic properties of the drug with high precision in time and space. As a consequence, the ability of the key to fit into the lock can be activated when and where we need it.

In many instances, we are not able to predict or explain the changes in biological activity that the “photoswitchable” drugs undergo when irradiated with light. In this thesis, we used advanced computer models to investigate several design principles for photopharmacology, with the ultimate goal of uncovering more rational ways to introduce switches into regular keys. We applied various methods to visualize and analyze the molecular interactions between the photoresponsive compounds and the biological target. These detailed models guided the design of drugs that could be specifically switched on with light, trying to minimize unexpected outcomes.

The calculations supported the choices of specific sites where we could introduce the molecular switch into the structure of a known drug. In particular, we focused on the azobenzene switch, which is converted from a stable, flat structure (the (*E*)-isomer) to a metastable, bent one (the (*Z*)-isomer) by means of light. The differences in the recognition of the two forms of the photo-drug (photo-keys) by the biological target (lock) were studied with an array of simulation techniques. Molecular docking predicts the orientation of the key inside the lock. Molecular dynamics goes beyond the static snapshot of docking and gives insights into the behavior of the key-lock system over time.

Throughout the thesis, we applied docking and molecular dynamics to understand and design keys that were fitting better in the lock upon irradiation. The investigated keys acted as photoresponsive anti-tumor agents (**Chapter 2**) and antibiotics (**Chapter 3**). With the same goal in mind, we also searched for subparts of known drugs that resembled the bent (*Z*)-form of azobenzene. Biaryl sulfonamides (**Chapter 4**) and benzophenones (**Chapter 5**) emerged as substructures suitable for these informed substitutions, yielding light-controlled drugs for the treatment of atherosclerosis and cancer (**Chapter 4**) or for the regulation of the circadian clock (**Chapter 5**). The rational design strategies described in this thesis will enter the toolbox of medicinal chemists in their quest for photoswitchable therapeutics.

7.4 Populaire samenvatting

De moderne geneeskunde heeft onze kwaliteit van leven verbeterd en het menselijk lijden verminderd door de ontdekking van talloze medicijnen. Telkens wanneer je een pil neemt, fungeert het actieve ingrediënt als een sleutel voor een specifiek slot in je lichaam (het biologische doelwit) dat verantwoordelijk is voor of verband houdt met uw ziekte. Nauwkeuriger gezegd, het doelwit past zich ook aan het binnenkomende medicijn aan, op dezelfde manier als een handschoen een hand omvat. Zodra het slot met de sleutel wordt geblokkeerd, activeert dit een biologisch effect dat de symptomen vermindert of de ziekte geneest.

Helaas veroorzaken medicijnen ook ongewenste bijwerkingen, omdat de sleutels interacties vormen met sloten in andere delen van het lichaam. Het opkomende gebied van fotofarmacologie probeert dergelijk gedrag op te lossen door medicijnen te ontwerpen die lokaal kunnen worden geactiveerd met licht. In een notendop, we modificeren het oorspronkelijke molecuul met een moleculaire fotoschakelaar, die kan worden geschakeld tussen "aan" en "uit" door bestraling met licht. Dankzij deze schakelaar kunnen we de 3D-vorm en de elektronische eigenschappen van het medicijn met hoge precisie in tijd en ruimte controleren. Als gevolg hiervan kan het vermogen van de moleculaire sleutel om in het slot te passen worden geactiveerd wanneer en waar we het nodig hebben.

In veel gevallen zijn we niet in staat om de veranderingen in biologische activiteit die de "fotoschakelbare" geneesmiddelen ondergaan wanneer ze met licht worden bestraald, te voorspellen of te verklaren. In dit proefschrift hebben we geavanceerde computermodellen gebruikt om verschillende ontwerpprincipes voor fotofarmacologie te onderzoeken, met als uiteindelijk doel het ontdekken van meer rationele manieren om lichtgevoelige

schakelaars in reguliere sleutels te introduceren. We hebben verschillende methoden toegepast om de moleculaire interacties tussen de fotoresponsieve verbindingen en het biologische doelwit te visualiseren en analyseren. Deze gedetailleerde modellen leidden het ontwerp van medicijnen die specifiek met licht konden worden aangeschakeld, in een poging onverwachte resultaten te minimaliseren.

De berekeningen ondersteunden de keuzes van specifieke locaties waar we de moleculaire schakelaar in de structuur van een bekend medicijn konden introduceren. In het bijzonder hebben we ons gericht op de azobenzeenschakelaar, die door middel van licht wordt omgezet van een stabiele, platte structuur (de (*E*)-isomeer) naar een metastabiele, gebogen (de (*Z*)-isomeer. De verschillen in de herkenning van de twee vormen van het fotodrug (foto-sleutels) door het biologische doelwit (slot) werden bestudeerd met een reeks simulatietechnieken. Molecular docking voorspelt de oriëntatie van de sleutel in het slot. Moleculaire dynamiek gaat verder dan de statische momentopname van docking en geeft inzicht in het gedrag van het key-lock-systeem in de loop van de tijd.

In het gehele proefschrift hebben we docking en moleculaire dynamica toegepast om sleutels beter te begrijpen en te ontwerpen die bij bestraling beter in het slot passen. De onderzochte sleutels werkten als fotoresponsieve anti-tumor middelen (**Hoofdstuk 2**) en antibiotica (**Hoofdstuk 3**). Met hetzelfde doel voor ogen zochten we ook naar onderdelen van bekende medicijnen die leken op de gebogen (*Z*)-vorm van een azobenzeen. Biarylsulfonamiden (**Hoofdstuk 4**) en benzofenonen (**Hoofdstuk 5**) kwamen naar voren als substructuren die geschikt zijn voor deze geïnformeerde substituties, wat lichtgestuurde geneesmiddelen opleverde voor de behandeling van atherosclerose en kanker (**Hoofdstuk 4**) of voor de regulatie van de circadiane klok (**Hoofdstuk 5**). De rationele ontwerpstrategieën die in dit proefschrift worden beschreven, zullen tot de gereedschapskist van medicinale chemici behoren en hun helpen in hun zoektocht naar fotoschakelbare geneesmiddelen en slimme therapieën.

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E quindi uscimmo a riveder le stelle.