UNIVERSIDADE D
COIMBRA

Rui Filipe Matias Mendes

Relatório de Estágio e Monografia intitulada "Ubiquitin Specific Protease 7 (USP7) Inhibitors as New Agents in Cancer Treatment" referentes à Unidade Curricular "Estágio", sob orientação da Dra. Teresa Cortez Nunes e do Professor Doutor Jorge Salvador apresentados à Faculdade de Farmácia da Universidade de Coimbra, para apreciação na prestação de provas públicas de Mestrado Integrado em Ciências Farmacêuticas.

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Eu, Rui Filipe Matias Mendes, estudante do Mestrado Integrado em Ciências Farmacêuticas, com o n.º 2014197310, declaro assumir toda a responsabilidade pelo conteúdo do Documento Relatório de Estágio e Monografia intitulada "Ubiquitin Specific Protease 7 (USP7) Inhibitors as New Agents in Cancer Treatment" apresentadas à Faculdade de Farmácia da Universidade de Coimbra, no âmbito da unidade de Estágio Curricular.

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Coimbra, 27 de outubro de 2020.

Rui Filipe Matias Mendes

(Rui Filipe Matias Mendes)

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Ao meu pai e à minha mãe por serem o meu suporte e ao meu irmão por estar na minha vida, obrigado por serem a minha casa, sem vocês nada seria possível.

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Obrigado!

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Capítulo I

Relatório de Estágio em Farmácia Comunitária

Sob orientação da Dra. Teresa Cortez Nunes

Lista de Abreviaturas

ANF - Associação Nacional das Farmácias

APPACDM - Associação Portuguesa de Pais e Amigos Do Cidadão Deficiente Mental

FC - Farmácia Cortesão

GAU - Gabinete de Apoio ao Utente

MICF - Mestrado Integrado em Ciências Farmacêuticas

MNSRM - Medicamentos Não Sujeitos a Receita Médica

PVF - Preço de Venda à Farmácia

PVP - Preço de Venda ao Público

SNS - Sistema Nacional de Saúde

SWOT - *Strengths, Weaknesses, Opportunities, Threats*

1. Introdução

O estabelecimento das práticas desenvolvidas pela farmácia comunitária como local de prestação de cuidados de saúde e pelo farmacêutico como profissional de saúde têm sido desde há muito tempo consideradas pelas comunidades como imprescindíveis para a promoção de saúde pública. A farmácia comunitária, mais do que um local de dispensa de medicamentos é, nos dias de hoje, um local de prestação de cuidados de saúde.[1] Assumindo-se assim como essencial e como um local de apoio constante que garante a qualidade, segurança e eficácia nas soluções, cuidados, serviços e aconselhamento que prestam e apresentam aos utentes que a procuram quer em caso de necessidade ou não.

A realização do Estágio Curricular em farmácia comunitária enquanto Unidade Curricular do Mestrado Integrado em Ciências Farmacêuticas (MICF) é um processo essencial à aprendizagem e desenvolvimento do aluno para o seu sucesso como futuro farmacêutico. Esta permite a consolidação e a aplicação dos conhecimentos da sua formação académica obtidos ao longo dos anos anteriores, para além de permitir a aquisição de novas competências e valências que apenas poderiam ser possíveis de ser esclarecidas e entendidas em contexto de situação real e profissional.

O meu estágio curricular decorreu na Farmácia Cortesão, na Rua do Padroeiro, na localidade de São Silvestre, concelho de Coimbra, sob a orientação da Dra. Teresa Cortez Nunes. Iniciou-se no dia 6 de janeiro de 2020 e terminou a 1 de agosto de 2020, tendo havido uma interrupção neste desde o dia 16 de março de 2020 até ao dia 5 de maio de 2020.

A Farmácia Cortesão encontra-se ao serviço da população há mais de um século, tendo já uma posição bem estabelecida entre a população local e não só, como um recurso fidedigno à saúde individual e familiar. Entre outros atributos a Farmácia é reputada pelo reconhecimento e valorização por parte dos utentes habituais, mas também pela empatia dos profissionais que a integram e apresentam para com os utentes onde num ambiente profissional, a proximidade, disponibilidade e preocupação funcionam como atributos favoráveis ao sucesso da Farmácia e à consideração dos seus utentes.

Este relatório terá assim como objetivo analisar criticamente o meu estágio curricular em farmácia comunitária sob a forma de uma análise *SWOT*, abordando os pontos fortes (*Strenghts*), os pontos fracos (*Weaknessess*), as oportunidades (*Opportunities*) e as ameaças (*Threats*) durante a realização de todo o estágio, permitindo uma análise das competências adquiridas e aspetos internos e externos que possam ter interferido na minha evolução profissional. Subsequentemente e incluído na análise *SWOT*, será referido o inevitável impacto do surto epidémico de COVID-19 na minha experiência.

2. Análise *SWOT*

2.1. Pontos Fortes

2.1.1. Localização e Horário

Como primeiro ponto, destaco a proximidade que a Farmácia tem com a estrada Nacional 111, aproximadamente a 300 metros. Esta estrada apresenta uma movimentação respeitável uma vez que liga Coimbra, a cidade de distrito, à Figueira da Foz, atravessando localidades com interesse demográfico, como por exemplo Montemor-o-Velho e Tentúgal, havendo dificilmente uma Farmácia tão próxima da estrada quanto esta, colocando-a num local de destaque e de fácil acesso, estrategicamente posicionada para uma vasta diversidade de utentes.

No entanto, o principal destaque que denoto da sua localização é o de ser a única farmácia edificada na freguesia de São Silvestre, concelho de Coimbra. Esta localidade poderá ser classificada, no meu entender, como uma região maioritariamente rural, todavia com zonas que já apresentam algum desenvolvimento urbano dentro da freguesia. Aliado a este, outro fator será também o de estar posicionada perto a uma área comercial, com considerável circulação diária de clientes, constituída por uma pastelaria com reconhecimento na zona, um hotel, um supermercado e outras lojas, criando assim oportunidades para potenciais utentes de visitarem o espaço da Farmácia.

Além desta proximidade com esta zona, atento que a Farmácia terá também como vantagem ter a presença de uma extensão de saúde na área de São Silvestre e, além disso, ter contactos estabelecidos com lares de idosos e com a instituição APPACDM (Associação Portuguesa de Pais e Amigos Do Cidadão Deficiente Mental) que se situam na região. Assim, através desta situação geográfica, considero que tive a oportunidade de contactar com utentes de todas as idades e com variados graus de literacia, permitindo realizar atendimentos bastante diversificados e personalizados. Observo assim que este foi um dos pontos fortes do meu estágio, na medida em que cada dia foi de constante aprendizagem individual e profissional.

Outro ponto forte do meu estágio na FC, que se alia à sua localização, é o horário de funcionamento da mesma. A FC encontra-se de serviço todos os dias úteis da semana das 9h até às 20h, e aos fins de semana apenas de manhã (sábado das 9h às 13h e domingo das 10h às 12h30). Este horário permitia assim que utentes, especialmente à população ativa, a oportunidade de recorrer à farmácia gerando um elevado número de casos reais.

Enquanto estagiário, reflito que este foi um ponto forte pois para além da variedade de atendimentos, deu-me a possibilidade de articular os meus horários confortavelmente uma vez que tive a necessidade durante o semestre de marcar presença em exames de primeiro

semestre em janeiro e fevereiro de 2020 e em aulas teórico-práticas de segundo semestre de quarto ano. Este horário manteve-se até à interrupção/pausa neste estágio e foi alterado devido à pandemia que obrigou a Farmácia a assegurar medidas para a contingência e prevenção ao contágio viral pela COVID-19, medidas essas que serão exploradas mais à frente neste relatório.

2.1.2. A Equipa e a Integração do Estagiário

Um dos pontos fortes que mais destaco no meu estágio curricular é a equipa técnica com que trabalhei, que desde o primeiro dia procurou orientar-me e auxiliar-me no dia a dia. A equipa da FC é composta por 5 elementos: a diretora técnica, duas farmacêuticas e dois técnicos de farmácia. Todos os elementos da equipa participaram no meu processo de aprendizagem durante o estágio, auxiliando quer na aplicação de conhecimentos teóricos quer na resolução dos mais variados desafios em que senti dificuldades.

Na FC, a equipa trabalha como um todo e em conjunto, considerando que as tarefas não são propriamente individualizadas. No entanto, os objetivos a cumprir diariamente estão previamente determinados e incutidos na equipa, salvaguardando sempre a intervenção e análise por parte da diretora técnica ou das farmacêuticas em caso de situações mais sensíveis ou em caso em que este o exija legalmente. Esta gestão de pessoal permite assim a maximização da capacidade de resposta da Farmácia às necessidades do cliente, uma vez que todos os elementos têm aptidão e adaptabilidade para qualquer questão diária que se coloque; e permite internamente que todas as tarefas diárias fundamentais possam ser concretizadas por cada um, de forma a atingir-se uma rentabilidade positiva em todas as vertentes dentro do que é o necessário às características da Farmácia.

Destaco, especialmente, a admirável capacidade de ajuste perante o surto epidémico que influenciou diretamente a equipa que se viu obrigada a ter de ser dividida, com o objetivo de que contacto e possível contágio fosse minimizado, quer para com os utentes de farmácia quer entre os próprios colegas, passando duas equipas a laborar em horários diferentes (o horário da farmácia foi assim estreitado e assumiu um horário para a manhã das 9h às 13h e um horário para tarde das 15h às 19h) e a assumir uma série de novas tarefas e medidas para o qual não estariam tão familiarizados tal como por exemplo proceder à desinfeção da farmácia duas vezes por dia após cada turno (entre as 13h às 15h e após as 19h e fecho da farmácia,) ou a preparação da logística e material específico antes do horário de abertura da Farmácia. Além das questões logísticas já mencionadas, também se procedeu a várias alterações físicas e visuais na Farmácia. Numa primeira instância e enquanto o país se manteve

em Estado de Emergência Nacional, o atendimento foi realizado através de um postigo colocado na entrada da farmácia podendo atender utentes a partir do meio exterior; após o Estado de emergência e segundo as ordens e iniciativas de desconfinamento promovidas pelos órgãos de poder de Portugal, a farmácia passou a permitir a entrada dentro do espaço, no entanto, mantendo e garantindo as medidas de segurança e prevenção.

Denoto assim que, através do meu estágio curricular, pude contactar com uma equipa completa, experiente e bastante determinada ao sucesso da Farmácia e à promoção do utente, e concluo que a equipa foi fundamental para o meu desenvolvimento pessoal e profissional, que fez questão de me acompanhar diariamente para que me sentisse suportado e integrado, permitindo e apoiando-me a tomar uma atitude confiante e independente na interação com os utentes e a prestar um serviço refletido e competente, promovendo a minha autonomia e ensinando-me a prestar um serviço enquanto futuro profissional de saúde na responsabilidade que assim o exige. Consequentemente a ocasião de ter estado na Farmácia durante uma pandemia também poderá ser considerado um ponto forte dado que tive oportunidade de lidar com situações e um acontecimento extraordinário comparado ao que é normal acontecer num estágio curricular. No entanto, considero também que terá sido uma ameaça, desenvolvendo este parecer mais à frente neste relatório.

2.1.3. Funções Desempenhadas

A diversidade de tarefas é outro ponto forte que posso destacar no meu estágio. A FC já se encontra em funcionamento há muitos anos pelo que já está adaptada a receber estagiários, o que me permitiu executar as tarefas diárias de maneira definida e por fases tendo a oportunidade de colaborar com toda a equipa. Tendo em conta que o trabalho de um farmacêutico numa farmácia comunitária não se prende apenas com o atendimento ao público, tive a oportunidade de realizar tarefas como: a receção e armazenamento de encomendas, compreendendo o impacto para a farmácia de uma boa gestão das encomendas, e, por conseguinte, dos *stocks*; serviços farmacêuticos de promoção de saúde (exemplo, análise de parâmetros bioquímicos), dinamização do espaço e preparação individualizada da medicação e ainda a preparação de manipulados.

2.1.3.1. Receção e Armazenamento de Encomendas

Nas primeiras semanas de estágio, o meu tempo útil restringiu-se à receção e posterior armazenamento das encomendas. Apesar de já ter tido feito um estágio de verão na mesma Farmácia, estas primeiras semanas foram importantes uma vez que me permitiu relembrar de

que maneira a Farmácia organiza os vários produtos e começar a relacionar as várias marcas e nomes comerciais a princípios ativos, indicações terapêuticas e às várias dosagens. Consequentemente, permitiu-me contactar e realizar encomendas diárias, geridas pelo SIFARMA 2000®, que vão de encontro com os *stocks* máximos e mínimos de cada produto, de encomendas instantâneas, ou produtos em falta. Tive ainda a oportunidade de relembrar e compreender novos termos e conceitos relacionados com o sistema operativo e com a organização e gestão de uma farmácia como por exemplo o preço de venda à farmácia (PVF) e o preço de venda ao público (PVP), o impacto das margens de lucro, a problemática dos medicamentos esgotados e o contacto com as diferentes distribuidoras farmacêuticas nacionais, entre outros.

Através destas tarefas, foi-me transmitido a importância no controlo de *stocks*, reconhecendo que estes devem ser compatíveis com as necessidades dos utentes, de forma a que o investimento tenha sustentabilidade e rentabilidade para a Farmácia. No seguimento da receção de encomenda dava-se o armazenamento dos produtos, um procedimento crucial que promove a velocidade de atendimento e a sua qualidade, tal como a gestão de stock. Assim entendi que para o sucesso da logística, funcionamento e organização da farmácia os produtos devem estar armazenados de maneira a serem facilmente localizados e dispostos segundo o princípio "*first expired, first out*", permitindo primeiramente o escoamento de produtos com prazo de validade mais curto.

2.1.3.2. Serviços Farmacêuticos de Promoção de Saúde

A Farmácia disponibiliza aos seus utentes o Gabinete de Apoio ao Utente (GAU), que é reservado para serviços farmacêuticos como medição de parâmetros bioquímicas e tensão arterial, e para consultas de Podologia e de Nutrição, dadas por especialistas que se deslocavam à farmácia em regime quinzenal. Ao longo do estágio pude comunicar com vários utentes no GAU num ambiente privado onde se criou a oportunidade de não só praticar as minhas técnicas de medição da tensão arterial, glicémia e colesterol total, como também contribuir para a consciencialização e promoção de práticas saudáveis entre os utentes como a alteração de hábitos alimentares e a prática de exercício físico. Com esta tarefa, considero que adquiri competências que me permitiram maior interação com o utente e aprimoraram as minhas técnicas de comunicação, através das quais comecei a detetar que muitas vezes alguns valores mais alterados se deviam a uma não aplicação de medidas não farmacológicas e a não adesão à terapêutica.

2.1.3.3. Atendimento ao Público

De todas as tarefas que desempenhei ao longo do estágio, o atendimento ao público foi inquestionavelmente a que tomou mais tempo deste e a que exigiu mais esforço. Desde cedo que a observação de atendimentos me foi incentivado por parte da equipa da FC, pelo que passei algumas horas ao lado dos mesmos a familiarizar-me com o programa, o discurso a ter com os utentes e a interpretação de receitas médicas. Durante esta visualização de atendimentos fui sempre motivado a manter um espírito crítico e a questionar qualquer eventualidade nas várias situações que presenciava, o que favorecia a consolidação de conhecimentos e as habilidades de comunicação. Após já ter ganho algum à vontade, comecei a realizar atendimentos com acompanhamento e só mais tarde me foi permitido a realização desta tarefa sozinho, com a segurança de que em caso de necessidade poderia solicitar o auxílio da equipa.

Transversalmente, entendi rapidamente que se tratava de uma atividade que impunha enorme concentração e responsabilidade, e que a manutenção de um contínuo espírito crítico é crucial, quer a nível científico, quer a nível dos seus aspetos legais, como por exemplo, na análise de uma receita médica em que há realmente a necessidade de explicar e reforçar a importância da adesão à terapêutica e cumprimento das indicações prestadas pelo médico ou pelo farmacêutico para que o medicamento possa ter efeito terapêutico esperado e que seja benéfico para o utente; relacionado a esta situação está o caso da dispensa de antibióticos em que alertava para o facto de ser necessário tomar a medicação sempre às mesmas horas e até ao fim da embalagem. Além dos esclarecimentos, colava nas embalagens as etiquetas redigidas com a posologia correta, com o objetivo de evitar esquecimentos ou dúvidas. Para além deste exemplo, também destaco outros grupos farmacológicos que são prescritos em grande quantidade como os anti-inflamatórios não esteroides, os inibidores da bomba de protões, as estatinas, entre outros, em que tinha o cuidado de informar acerca das particularidades dependendo do tipo de medicamento.

Para além da dispensa de receitas médicas, em casos onde seria necessário um aconselhamento farmacêutico, o meu primeiro objetivo era recolha de informações acerca da pessoa em atendimento e foi um dos pontos em que me deparei com mais dificuldade no atendimento ao público. No entanto, entendi a importância destas questões ao utente e na qualidade de atendimento que produzia, apercebendo-me que quanto mais informação obtivesse, melhores e mais rápidas soluções encontrava uma vez que me permitia uma análise o mais completa possível da situação, prevenindo inconvenientes e descontentamento por parte do utente. Assim, trabalhei no sentido de conseguir ultrapassar este obstáculo, pois só

desta maneira seria possível, no meu ponto de vista, aumentar a satisfação do utente levando-o a sentir-se seguro e relevante; e a promover adesão à terapêutica, a fidelização à farmácia e a minha progressão enquanto futuro farmacêutico. Após esta recolha de informação, prosseguia o meu atendimento, que se baseava no seguinte: inicialmente no aconselhamento de medidas não farmacológicas, caso não houvesse necessidade de introduzir um medicamento não sujeito a receita médica (MNSRM); caso houvesse, apresentava assim as várias opções, tendo em conta a relação benefício-risco-custo e explicava a posologia que considerava mais segura e eficaz, procurando sempre promover o princípio do uso racional do medicamento; em casos em que se tratasse de uma situação mais complicada, aconselhava o utente a dirigir-se ao médico. Concluo assim este ponto afirmando que o atendimento se revelou um desafio diário que procurei sempre melhorar, tentando assimilar o que me era ensinado de novo, aplicar os meus conhecimentos previamente adquiridos e mostrar interesse e empatia para com o utente, adaptando-me a cada caso.

2.1.4. Dinamização e Gestão do Espaço

A organização e gestão do espaço da farmácia é promovida pela equipa de forma a ser acolhedora, apeladora e com boa visibilidade e sinalização para os vários setores, marcas e produtos explorados. As montras e outros locais de exposição de produtos da farmácia são reformulados regularmente, de maneira a atualizar os utentes acerca das promoções a decorrer. Desta maneira, consegui visualizar e contribuir para a elaboração destes locais de exposição, permitindo aplicar conhecimentos de *marketing* e organização e gestão farmacêutica, pelo que considero que esta atividade também foi um ponto forte do meu estágio.

2.1.5. Fidelização e Utentes

A diversidade de utentes que tive a oportunidade de contactar foi outro dos pontos fortes que realço no meu estágio na FC. Embora se constituísse maioritariamente por população idosa, polimedicados e utentes com doenças crónicas, os utentes de outras faixas etárias, desde recém-nascidos, a crianças e adultos também participavam desta diversidade, estando similarmente fidelizados à farmácia. Deste modo, a heterogeneidade da população contribuiu para que o meu processo de aprendizagem fosse contínuo e que quase diariamente surgissem situações novas.

Sendo a satisfação do utente uma das máximas da FC, esta é reconhecida pelo apoio constante que fornece aos seus utentes e pela preocupação transmitida, quer nas várias

tentativas imediatas de arranjar medicamentos indispensáveis, quer na disponibilidade demonstrada para os fazer chegar no menor intervalo de tempo possível ao respetivo utente. Enquanto estagiário, procurei sempre agradar ao utente com o serviço prestado seguindo as máximas da Farmácia e os concelhos da equipa, tendo para além disso, incentivado a criação de novas fichas de utente no SIFARMA 2000®, o que facilita o acesso ao historial de venda de produtos à pessoa em questão, trazendo inúmeras vantagens, nomeadamente em termos dos medicamentos e respetivos laboratórios que tem por hábito levar, informação acerca de planos de comparticipação; o acesso ao cartão Saúda (ao qual também estimulei utentes à sua adesão, uma vez que beneficiariam deste ao terem direito a descontos e/ou a ofertas de produtos em troca de pontos acumulados por compras efetuadas na própria farmácia e em todas as associadas às Farmácias Portuguesas) se previamente associado, e acesso a informações pessoais como o n.º de contribuinte, entre outras informações convenientes.[2] Considero assim, que estas ações trouxeram diversas vantagens para a farmácia, mas também para o utente uma vez que este sabe que na farmácia em questão há registo dos seus produtos e acesso facilitado aos seus diversos dados, evitando a repetição de perguntas e prolongamento do atendimento. Assim considero este um ponto forte pois, para além das novas ferramentas com que me familiarizei, consegui colaborar para a satisfação do utente.

2.2. Pontos Fracos

2.2.1. Descredibilização por Parte dos Utentes

Devido ao meu estatuto na farmácia como estagiário em que o meu principal objetivo foi de aprendizagem, a maior parte dos utentes tentavam ajudar-me, como por exemplo ao alertar-me para o facto de deterem planos de complementaridade com o Sistema Nacional de Saúde (SNS) ou outros. Porém, nem todos os utentes se sentiam à vontade para ser atendidos por mim devido a não ter tanta experiência e por vezes solicitavam ser atendidos por um membro efetivo, ou outro membro da equipa em específico, negando o atendimento por parte do estagiário que não os conhece há muito tempo. Reflito que será um ponto fraco uma vez que não tive a oportunidade de desenvolver ou encontrar soluções de forma independente para uma questão que possivelmente poderia ter capacidade de resolver, acabando por perder essa experiência e não desenvolver a minha autonomia e responsabilidade profissional.

2.2.2. Poucas Formações

A Farmácia Cortesão reconhece-se como uma farmácia que prima por estar a par das últimas novidades dentro das várias áreas que explora, e incentiva diariamente a equipa para a continua atualização e formação acerca de novos produtos e realização de formações individuais e em grupo. Infelizmente, durante o meu estágio não houve oportunidade de participar em muitas formações devido à pandemia, tendo, no entanto, ainda participado em algumas atividades relacionadas que decorreram fora do espaço da Farmácia antes do pico da pandemia. Estas foram a formação promovida pela *Pharma Nord* acerca dos Suplementos alimentares *BioActivo*®, e a conferência "Coronavírus 2019-nCoV- Ameaça sem Fronteiras - O essencial para saber aconselhar!" promovida pela Associação Nacional das Farmácias (ANF) sobre alguns esclarecimentos de prevenção e aconselhamento à COVID-19. Relativamente a formações internas, decorreram na própria Farmácia, apresentadas por delegados das respetivas marcas, às quais pude marcar presença, como por exemplo da gama de produtos da" Tilman", da "Alpecin", entre outras.

As formações que presenciei permitiram-me recordar alguns conhecimentos do ponto de vista científico e conhecer diversos produtos de diferentes marcas, bem como as últimas novidades no mercado, colaborando para o aperfeiçoamento das minhas técnicas de venda, quer de *cross-selling* quer de *up-selling*. Assim, admito que as poucas formações foram um ponto fraco relativamente ao meu estágio, uma vez que teria sido vantajoso a realização de mais formações durante o meu estágio curricular de maneira a complementar a minha formação acerca das mais variadas marcas e áreas de saúde enquanto futuro profissional de saúde.

2.3. Oportunidades

2.3.1. Preparação de Medicamentos Manipulados

Uma oportunidade que destaco no meu estágio na FC foi o facto de ter realizado medicamentos manipulados. De facto, a evolução da indústria farmacêutica faz com que o número de pedidos deste tipo de medicamentos que chega à farmácia seja cada vez mais reduzida. No entanto, enquanto estive na FC preparei alguns medicamentos manipulados tal como a solução alcoólica de ácido bórico à saturação, utilizado devido ao seu efeito antissético e desinfetante para tratar otites externas, e ainda uma pomada de vaselina enxofrada para o tratamento da escabiose, mais popularmente conhecida como sarna. Considero, então uma oportunidade, uma vez que nem todas as farmácias costumam ter estes serviços e a procura destes pedidos seja, no seu geral, diminuída.

2.3.2. Dermocosmética, Veterinária e Suplementos Alimentares

Quando nos referimos a todas as áreas e marcas exploradas pelas farmácias comunitárias nos dias de hoje, poderemos certamente caracterizá-las como numerosas e distintas, o que implica que para a satisfação dos clientes nas várias áreas, a diversidade de produtos na farmácia seja imensa com o objetivo de corresponder com todas as necessidades dos utentes. Inevitavelmente, na formação teórica nos anos anteriores ao estágio curricular, não foi então possível aprofundar e conhecer toda esta variedade. Considero assim uma oportunidade o facto de ter adquirido e complementado a minha formação com novos conhecimentos em áreas como a dermocosmética, veterinária e suplementos alimentares, uma vez que a FC possuía grandes lineares destas áreas, com várias marcas e produtos que eram regularmente solicitados por utentes. Admito que, porém, inicialmente tenha sentido uma grande dificuldade em aconselhá-los, com o auxílio da equipa técnica da FC, algum estudo e esforço para reter o máximo de informação possível, comecei a conseguir aconselhar algumas marcas e produtos com alguma confiança e destreza. Desta forma, pude contactar e conhecer melhor os produtos destes âmbitos, facilitando associação e aplicação dos conhecimentos teóricos adquiridos à realidade.

2.4. Ameaças

2.4.1. COVID-19

Inquestionavelmente, destaco a pandemia de COVID-19 e as implicações relacionadas com a minha permanência no estágio a principal ameaça a este. Primeiramente, porque ao estagiar numa farmácia com uma movimentação constante de utentes e ao exercer o atendimento ao público, estaria inevitável exposto ao risco de ser contagiado. Seguidamente, porque obrigou à interrupção do estágio desde o dia 16 de março até ao dia 5 de maio de 2020, devido às medidas que aconselharam a população a realizar quarentena em razão do risco de contágio pelo vírus e ao Estado de Emergência Nacional decretado pelos órgãos de poder. Fui assim obrigado a adiar o final do estágio até agosto, o que impediu que realizasse um segundo estágio em Indústria Farmacêutica que estaria projetado para se iniciar em maio. Esta interrupção acabou por levar a várias consequências, iniciando pela diminuição do nível de motivação relativamente à continuação do estágio devido a ter perdido a oportunidade de realização do segundo estágio curricular, causado pela ameaça à saúde pública. Esta contrariedade fez com que, para acabar o estágio curricular em farmácia comunitária, as horas mínimas estabelecidas aumentassem e prolongassem o meu estágio até agosto de 2020. Além desta, a interrupção fez-me perder os hábitos de rotina, aliado aos horários da própria

farmácia que mudaram (tendo sido reduzidos e a equipa dividida em turnos), e dificultou a minha *performance* na execução de tarefas ao qual já estava acostumado a lidar diariamente na farmácia antes da interrupção, tendo levado algum tempo até ter voltado a ganhar o ritmo de trabalho perdido. Concluo assim este ponto como a maior ameaça porque realmente impediu que tivesse uma aprendizagem contínua ao longo do tempo e transversalmente de trabalhar com a equipa na sua totalidade tendo impactado inevitavelmente na minha prestação enquanto estagiário na Farmácia Cortesão.

2.4.2. Plano de Estudos do MICF Incompleto

Entre as ameaças que identifico, reconheço que o facto de ter realizado o meu estágio curricular sem antes completar algumas disciplinas do plano curricular do MICF na sua totalidade, devido a não ter créditos suficientes para me inscrever a cadeiras de 5° ano, poderão provavelmente ter impactado ou até dificultado a minha experiência com o decorrer do meu estágio na medida que poderei ter tido falta de *background* teórico para determinados produtos dispensados e explorados na farmácia comunitária, como o caso das unidades curriculares de Preparações de Uso Veterinário e/ou de Fitoterapia. Prevenindo essa ameaça, procurei autonomamente preparar e adaptar-me pesquisando mais acerca destas temáticas e pedindo conselhos à equipa da Farmácia Cortesão, aliando à particular atenção que prestei a atendimentos que envolvessem produtos que desconhecia ou não estava tão familiarizado.

2.4.3. *Stocks* e Medicamentos Esgotados

No decorrer do meu estágio curricular, foram vários os medicamentos que se encontravam esgotados nas farmácias e em todos os armazenistas e fornecedores, uma situação que infelizmente não é tão incomum nos dias de hoje. Considero que esta situação tenha sido uma ameaça ao estágio uma vez que é sempre uma interação negativa para com o utente pois não consegue adquirir o produto que procura, adicionando ao facto de alguns utentes não compreenderem o conceito de esgotado, culpando muitas vezes a farmácia por "deixar esgotar o produto".

Especial destaque ao período anterior ao pico da pandemia em Portugal que causou uma procura exacerbada por parte dos utentes de medicamentos como anti-inflamatórios não-esteroides, de material de desinfeção como álcool-gel, e material de proteção individual como máscaras e luvas descartáveis, o que causou o esgotamento de *stocks* a nível nacional e ainda mais pânico generalizado pela população. Para um estagiário, muitas vezes pela estranheza que já mencionei acima, nem sempre é fácil explicar que a falta do medicamento,

ou neste destaque de produtos específicos, não é da competência da farmácia que este representa, situação que resultou, em algumas ocasiões, na necessidade de intervenção de elementos da equipa mais experiente para reforçar o esclarecimento. Apesar de considerar uma ameaça, esta também poderá ser idealizada como uma oportunidade para a promoção de "ações de educação dirigida à comunidade no âmbito da promoção da saúde".[1]

3. Considerações Finais

Com a minha participação neste estágio e a vivência da realidade do que é o dia a dia numa farmácia, aliado às circunstâncias extraordinárias proporcionadas pela pandemia, decerto que considero que o estágio foi uma experiência que me desenvolveu académica e pessoalmente. Primeiramente, ao reforçar a minha ideia acerca da posição do farmacêutico na comunidade, um profissional ao qual os utentes recorrem como solução fiável e de confiança, que se demonstra e está facilmente disponível para ajudar qualquer indivíduo em qualquer problema do quotidiano sem constrangimentos nem preconceitos. Por esta razão, percebo que será de extrema relevância, de maneira a continuar a merecer esta confiança, que o farmacêutico invista, ao longo da sua carreira profissional, na sua formação e atualização técnica e científica continuamente, desenvolvendo e valorizando consequentemente o que é a farmácia para a comunidade, apostando cada vez mais na melhoria de serviços farmacêuticos e sua personalização de forma a criar e melhorar a relação utente-farmacêutico e a promoção de saúde pública, pois é nas farmácias que são feitas muitas das primeiras avaliações ao doente e se transmite a importância de praticar um estilo de vida saudável na prevenção de doenças.

Para finalizar, deixo um enorme agradecimento à equipa da FC, que me integrou na sua rotina e ensinou-me tudo o que estava ao seu alcance, contribuindo para o meu desenvolvimento profissional e pessoal. Termino o meu estágio com a certeza de que com a minha participação neste me tornei certamente um profissional de saúde mais capaz ao adquirir novos e aprimorados valores e capacidades que me deixam preparado para encarar o futuro e seus desafios, e, não menos importante, me tornou conhecedor da relevância da prática farmacêutica para as comunidades e da responsabilidade e competência que esta o impõe.

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5. Anexos

5.1. Casos Clínicos

Caso 1: Uma utente do sexo feminino com idade aparente de 50 anos, dirige-se à farmácia com o objetivo de adquirir um medicamento sujeito a receita médica. Na receita médica encontrava-se uma linha com a prescrição de topiramato 50 mg, ao qual a senhora pediu que lhe fosse dispensada duas caixas. No entanto, pretendia que estas fossem de formulações diferentes, ou seja, uma caixa na formulação de cápsula e outra na de comprimido revestido por película.

O topiramato é indicado em adultos para a profilaxia da enxaqueca, após avaliação cuidadosa de possíveis opções alternativas de tratamento, não sendo indicado para casos de tratamento agudo.[3,4]

Ao questionar a senhora entendi que a razão do pedido se devia ao facto de que a receita e o medicamento prescrito serviam tanto para a senhora em atendimento como para a sua filha. No entanto, a utente informou que não se dava bem com as cápsulas, já a filha não teria qualquer inconveniente com esta formulação. Por esta razão, a senhora costumava pedir na formulação de comprimidos revestidos por película, alertando para o facto de que costuma levar uma marca especifica uma vez que é extremamente intolerante à lactose.

Após ter identificado a marca através do histórico de compras da utente e verificado que nos excipientes deste não continha lactose, confirmei que esta marca se encontrava esgotada e que não havia em *stock*.[4] Comuniquei assim estas informações à utente e pedi que me desse mais algum tempo para analisar e verificar no sistema se haveria outra marca que também não tivesse na sua formulação a lactose, concluindo que de facto não existia. Adicionalmente a esta pesquisa, tentei ainda ligar aos armazenistas ao qual também me indicaram que de facto não conseguiam saber se haveria algum medicamento com estas características específicas.

Terminei o atendimento ao relatar à utente que infelizmente não conseguiria ter na minha posse nem encomendar o medicamento com as características pretendidas, aconselhando a senhora a ligar para a linha telefónica gratuita 1400, denominada por Linha de Apoio Farmacêutico (LAF) que permite a utentes encomendar medicamentos que necessitem. Por fim, a utente acabou por levar apenas a caixa com a formulação em cápsulas que já pretendia levar a para a filha e que acabaria também por tomar desta apesar de não ser o mais confortável.

Caso 2: Uma utente do sexo feminino, idosa, polimedicada, dirige-se à farmácia para ir buscar a sua medicação habitual. Entre vários medicamentos, a utente pediu se lhe podia dispensar uma caixa de Velmetia®, um antidiabético oral composto por uma associação entre sitagliptina e metformina, na dose de 50 mg/1000 mg, sem receita médica.[5] Devido a ser uma cliente habitual, ao consultar o histórico de compras da utente, pude confirmar que nunca adquiriu na FC este medicamento, deparando-me que relacionado com antidiabéticos orais a utente apenas tomava o Eucreas® 50 mg/850 mg, uma associação entre vildagliptina e metformina que já adquirira múltiplas vezes na FC com receita médica.[6]

Perguntei então à utente o porquê de querer levar este medicamento uma vez que não era habitual nem trazia receita médica deste, ao que me respondeu que lhe foi aconselhada pelo irmão, também diabético, que lhe teria dito que com este medicamento conseguia manter os valores da glicémia conformes, e dado que de que a utente não teria, iria experimentar levar este novo medicamento.

Após esta explicação, transmiti à senhora que não lhe poderia dar o medicamento pretendido uma vez que não fazia parte da sua terapêutica, e além disso tentei dar a entender à utente de que essa alteração não era a mais acertada não lhe podendo dar o medicamento, pois a decisão de mudança de medicação e/ou aumento da dose neste tipo de medicação cabia ao seu médico prescritor, explicando ainda os riscos e consequências associadas ao aumento da dose como o caso de acidose lática em situações de sobredosagem de metformina, e por fim alertei que a toma da medicação de forma continuada e a horas a poderia ajudar a manter os seus níveis ajustados, e, se realmente, caso não obtivesse melhores resultados, aconselharia a sua ida ao médico, para uma nova avaliação da terapêutica.[5,6]

Capítulo II

Monografia

Ubiquitin Specific Protease 7 (USP7) Inhibitors as New Agents in Cancer Treatment

Sob orientação do Professor Doutor Jorge Salvador

List of Abreviations

AKT – Protein kinase B

ATR – Ataxia-telangiectasia mutated and Rad3-related kinase

Chk1 – Checkpoint kinase 1

DDR – DNA damage response

DUB – Deubiquitinase

E1 – Ubiquitin activating enzyme

E2 – Ubiquitin conjugating enzyme

E3 – Ubiquitin ligating enzyme

Gly – Glycine

HAUSP – Herpesvirus- associated ubiquitin specific protease

Lys – Lysine

MDM2 – Mouse double minute 2 homolog

MM – Multiple myeloma

PIP_3 – Phosphatidylinositol-3, 4, 5-triphosphate

PTEN – Phosphate and tensin homolog

PTM – Post-translational modifications

SPR – Surface plasmon resonance

TRAF – Tumour necrosis factor receptor-associated factors

Ub – Ubiquitin

Ub-PLA2 – Ubiquitin phospholipase A2 enzyme assay

UPS – Ubiquitin-proteasome system

USP – Ubiquitin specific protease

Resumo

Associado aos esforços para compreender a complexidade do cancro como doença e alcançar melhores resultados para superá-lo clinicamente, mas também cientificamente, estão a ser investigadas novas moléculas capazes de influenciar mecanismos celulares para alcançar melhores soluções terapêuticas do que as atuais. O sistema ubiquitina-proteassoma é um mecanismo celular frequentemente utilizado pela investigação por ser considerado um regulador chave de degradação proteica a nível celular, sendo composto por dois componentes principais: a ubiquitina e o proteassoma. Proteínas marcadas com ubiquitina normalmente são determinadas a sofrerem degradação no proteassoma, apesar de outros destinos serem possíveis. Por esta razão, poderá ser presumido que este sistema deverá ter um papel na regulação de proteínas ou de fatores de transcrição conectados ao processo de carcinogénese, como a p53 (uma proteína "guardiã" do genoma que facilita a eliminação de células em risco de mitose aberrante) e outros intervenientes relacionados. A marcação de ubiquitina pode ser revertida pela ação de enzimas especializadas, as deubiquitinases, capazes de evitar que os substratos marcados sejam degradados. Há diferentes tipos e famílias destas enzimas especializadas, enfatizando a família das protease especifica de ubiquitina ("ubiquitin-specific proteases") e mais especificamente a USP7 (ubiquitin specific protease 7) que tem atraído muita atenção por parte dos investigadores, tendo já em desenvolvimento um número considerável de potenciais novas moléculas que demostraram inibi-la, os inibidores da USP7, que têm obtido resultados otimistas quanto à supressão de várias linhas celulares carcinogénicas e de outros fatores de progressão de carcinogénese.

Palavras-chave: Cancro, Sistema Ubiquitina-Proteassoma, Deubiquitinases, USP7, Inibidores USP7.

Abstract

Associated with the efforts to comprehend the complexity of cancer as a disease and reaching for better outcomes in the path to overcoming it clinically, but also scientifically, new small molecules able to influence cell mechanisms are being targeted to reach better therapeutic solutions than the current ones. The ubiquitin-proteasome system is a cellular mechanism frequently employed in research for being considered a key regulator of protein degradation in cells, composed of two major components: the ubiquitin and the proteasome. Proteins tagged by ubiquitin (Ub) usually end up degraded in the proteasome, although other outcomes are possible. For this reason, it can be presumed that this system should have a role in the regulation of critical proteins or transcription factors connected with tumorigenesis process, such as p53 (a genome safeguarding protein which facilitates the elimination of cells at risk of aberrant mitoses) and others. The ubiquitin tagging can be reversed by specialized enzymes, deubiquitinases, able to prevent the marked substrates from degradation. There are different types and families of these specialized enzymes, emphasizing the family of ubiquitin-specific proteases and more specifically USP7 (ubiquitin specific protease 7) which has attracted a lot of interest from researchers, with a considerable number of new small molecules exhibiting potential to inhibit it in development, the USP7 inhibitors, which are getting optimistic outcomes in supressing carcinogenic cells lines, and other progression factors of tumorigenesis.

Keywords: Cancer, Ubiquitin-Proteasome System, Deubiquitinases, USP7, USP7 inhibitors.

1. Introduction

Throughout human evolution, society had constantly undergone numerous challenges and complexities, especially regarding health issues. Although some challenges may be overcome, new ones arise. One illustration is how over the last few decades, with the overall scientific and human development, conditions like better sanitation, antibiotics and vaccine development have brought a decline in mortality from infectious diseases.[1] In addition, improvements on cardiovascular diseases prevention, demographic and epidemiologic transitions have led to today's observation that cancer is the first or second leading cause of premature death (i.e. at ages 30–69 years) in 134 of 183 countries and it ranks as third or fourth in an additional 45 countries.[1]

Cancer as a disease can be considered complex and its patterns can vary a lot between specific cancer types and countries, since every individual can have innate determinants or be affected by local exposures to conditions or determinants that can cause it.[1] Over the last few decades, efforts have been made in order to understand cancer and its characteristics with the objective of overcoming it as a human health challenge.[1] Due to this, extensive research has revealed a small molecule, ubiquitin (Ub), capable of carrying out an elaborate control of a variety of cellular processes.

Post-translational modifications (PTMs) exponentially increase the complexity and functional diversity of the human proteome, allowing rapid and dynamic responses to variable environmental and intracellular conditions. Simultaneously, it is crucial for cells to have and maintain a robust regulation of the proteome for a correct interaction between different proteins necessary for a normal cellular function, survival, and proliferation.[2,3] There are numerous articles about new possible molecular targets for cancer treatment and several cell mechanisms are currently being studied. This article will focus on the ubiquitin-proteasome system (UPS) which plays a crucial role for the protein homeostasis in cells as a key regulator of the eukaryotic protein-degradation machinery.[3,4,5] UPS is composed by a tagging factor, the Ub, a molecule that marks unwanted or damaged proteins for degradation; and the proteasome, responsible for the disintegration of these tagged proteins into small peptides. More than 80% of cellular proteins are degraded through this pathway, exalting the importance of UPS in the regulation of cellular processes.[3,4,5] Recognized as the molecular shredder of UPS, the 26S proteasome is an ATP-dependent multi-subunit proteolytic structure found in the nucleus and in the cytoplasm of eukaryotic cells. It can be separated into two groups of sub-structures: a catalytic 20S core particle, containing the necessary protease active sites, and

two 19S regulatory particles, which function as a selective facilitator in transfer to the 20S core particle.[3,4,5]

UPS is involved in, and with a diversity of essential cell procedures and biological agents, and can be reflected as a cellular machinery that has multifaceted roles, comprehending processes like the degradation of misfolded and damaged proteins, cell cycle regulators, oncogene and tumour suppressor proteins, as well as the regulation of antigen processing and control of transcription factors activity. Thus, by consideration of the diversity of UPS substrates and their potential, it is no surprise that this pathway has been connected to the pathogenesis of several human diseases related with neurodegenerative conditions, viral diseases, cancer and more.[5]

2. Ubiquitin and ubiquitination

Ub is a small, highly compacted globular protein and a greatly conserved 76 amino acid protein, except for its unrestrained and flexible C-terminal tail. The process of ubiquitination is a multi-step process and is achieved by an organized and sequential enzymatic cascade, leading to the covalent modification of a protein substrate with the addition of a small molecule of ubiquitin. Characteristically, the process of ubiquitination is dependent on the consecutive activity of three recognizable enzymes: Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3).[2,5]

Triggered by an ATP-dependent reaction and by an E1 enzyme, a thioester bond is formed between the carboxy-terminal glycine (Gly) residue of ubiquitin and the active site cysteine of the E1 enzyme. Subsequently, ubiquitin is transferred from E1 to the active cysteine residue of one of the E2 enzymes followed by the Ub attachment to a substrate, mediated by an E3 enzyme. Substrate specificity is conferred by E3 ligases, which bind target substrates and coordinate the covalent attachment of ubiquitin. So far, 2 E1s, nearly 30 E2s and over 600 E3s have been identified in humans. The ubiquitination is completed when the covalent bond between the α–carboxyl group of the terminal glycine residue of Ub and, typically, an ε-amino group of an internal lysine (Lys) residue of a target protein is formed.[2,5]

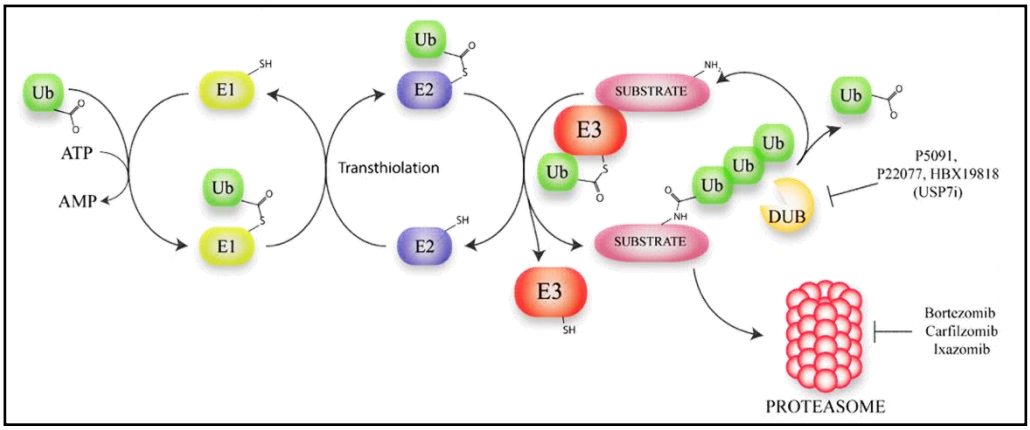


Figure 1 - Scheme of ubiquitin pathway and USP7 inhibitors placement in it. (Adapted from Wu *et al.*)[6]

In a mechanical way, 3 classes of E3 ligases can be described: RING-type ligases, HECT-type ligases, and RBR-type ligases. E3 ligases select the substrate and promote ubiquitin transfer onto the target either directly from the E2 conjugating enzyme, a mechanism adopted by (Really Interesting New Gene) RING-type ligases, or in other way, via an E3-ubiquitin thioester intermediate as detected in (Homologous to E6-AP C terminus) HECT-type ligases and the (RING-between-RING) RBR-type ligases (hybrid from RING and HECT ligases).[2,7]

Normally, proteins are marked for proteasomal degradation via post-translational modification with ubiquitin, however the addition of ubiquitin regulates a multitude of diverse cellular processes and functions. These include subcellular localization, protein sorting, and protein-protein interactions (PPIs), with the protein substrates themselves ultimately subjected to different actions like protein activation, inhibition, as well as proteasomal or lysosomal degradation, depending on their ubiquitination pattern.[3]

Ubiquitination cannot be considered a linear sequence of reactions since it can occur in various structural shapes. This structure is usually referred to as the "Ub code". When the ubiquitinatination involves a single lysine of a targeted substrate with a single Ub it materializes into a monoubiquitination, the amplest Ub modification that regulates DNA repair, transcription, signal transduction, viral budding, endocytosis and even proteasomal degradation. After the first Ub transfer to the ε-amino group of a target Lys, any of the eight amino groups of Ub (Met1, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) can serve as sites for the covalent attachment of other ubiquitin molecules, being attached to the C terminus of another Ub to form a Ub chain of mutable length and configuration (homo- and heterotypic/branched Ub chains) which can be classified as multimonoubiquitination (single ubiquitins on multiple lysines) or as polyubiquitination by the formation of ubiquitin chains.[2,3,5]

The sequential type of ubiquitin within the polyubiquitin chain determines and can provide critical data on the fate of the conjoined protein. An example of it are the tagged proteins with Lys48-linked polyubiquitin chains which are usually fated to proteasomal degradation or the modifications involving the Lys63-linked chains, typically associated with nonproteasomal roles such as DNA repair, DNA replication and signal transduction.[2,3,5]

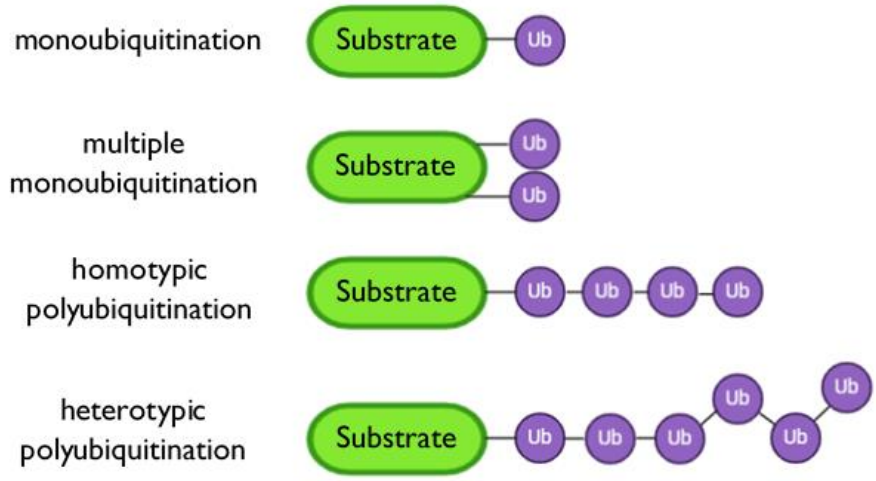


Figure 2 - Ub molecules undergo various PTMs, including phosphorylation and acetylation, interfering with their binding properties and abilities to generate Ub chains. Single Ub moieties can modify proteins at one (monoubiquitination) or several (multiple monoubiquitination) residues. Additional complexity is achieved through the formation of heterotypic Ub chains, which contain multiple Ub linkages and adopt mixed or branched topology. (Adapted from Kliza *et al.*)[2]

3. Deubiquitinases

Ubiquitination is a remarkable dynamic process and can be turned back by the action of specialized enzymes known as deubiquitinases (DUBs). These enzymes have a mechanism that reverses the E3 ligases action by promoting the separation of the isopeptide bond between lysine residues on target proteins and the C-terminal glycine of Ub, resulting in the Ub's deconjugation from the ubiquitinated protein. As a result of DUBs activity, the cellular pool of free Ub is affected by the release of newly synthesized Ub from Ub precursors, removing non-essential Ub molecules and recycling Ub from the former ubiquitination events.[2,5,8]

The human genome can encode approximately 100 DUBs and the function of the majority still remains to be determined, however there is evidence that deubiquitination of mono-Ub or poly-Ub chains can induce altered protein's localization, trafficking, or enhanced stability. Generally, DUBs are presumed to exist as free enzymes, but some have been

identified through their association with larger enzyme complexes. Considering the total number of DUBs identified and the degree of variability within the different family members, they are considered highly druggable, with a set of DUB inhibitors currently in various stages of pre-clinical development.[2,4,5,8]

DUBS can be divided into two main classes taking into account the enzymatic cleavage mechanism: cysteine proteases and zinc metalloproteases; but when classified according to sequential and structural similarity, they are distributed into six subfamilies: ubiquitin-specific proteases (USPs), monocyte chemotactic protein-induced protein (MCPIP), Machado–Joseph disease protein domain proteases (MJDs), ovarian-tumor proteases (OTUs), ubiquitin carboxyterminal hydrolases (UCHs), and JAMM/MPN domain-associated metallopeptidases (JAMMs). JAMMS is the exception, belonging to the metalloprotease's family. All the others are considered cysteine proteases.[4,9]

Among all the DUB subfamilies, the USP family is the largest one, with approximately 60 members, with sizes ranging from 50 to 300 kDa, which, albeit being different, all contain conserved domains–three major functional domains of Cys, His and Asp boxes–which are in charge of conjugated ubiquitin molecules. Within such a wide range of USPs, it is reasonable to speculate that there must be a panoply of mechanisms. Indeed, different USPs have their diverse substrate specificity, modification preference and regulation mechanism, supported by the evaluation of gene mutations and aberrant expression of USPs in various cancers. USPs are considered potential anticancer targets, and there is an increasing interest in developing USP-specific inhibitors as contenders for new anticancer therapeutic agents. These characteristics made USPs become increasingly appreciated for research in a variety of diseases.[4,9]

4. USP7

Several authors consider that among all the USP members, USP7 is arguably the most prominent and well characterized one due to being one of the first therapeutically related DUBs as well as one of the most actively sought after DUB targets in cancer drug discovery. In early research, USP7 was recognized for its strong interaction partner, an E3 identified as ICP0, which is a powerful regulatory protein of herpes simplex virus type 1 (HSV-1). The enzyme thus received its original name "Herpesvirus-associated ubiquitin specific protease" (HAUSP), and later became officially known as USP7.[9]

A lot of articles had described the correlation between the USP7 and the oncogenic processes of cancer's development such as the direct association of USP7's levels with the

tumorigenesis evolution on prostate cancer, ovarian cancer, multiple myeloma (MM), etc. This USP7 expression can be associated with cancer stages, tumour sizes and prognosis, which reinforces the potential that USP7 may have as a prognostic marker in certain cancers. There is a plethora of information concerning other examples of associations, moreover, there is a particular one where patients with elevated USP7 levels exhibited an inferior overall survival rate in comparison with patients with lower USP7 levels.[4]

4.1. USP7 Structure

USP7 contains 1102 residues in its full length and comprehends four domains: a TRAF-like domain in its N-terminus (USP7-NTD), constituted by a N-terminal poly-glutamine stretch (Poly Q) associated to a tumour necrosis factor receptor-associated factors (TRAF) domain; a catalytic domain, a 5-ubiquitin-like (UBL) domain, also named HAUSP ubiquitin-like (HUBL) domain; and a C-terminal regulatory domain (CTD).[9,10,11]

The first amino-acid residues (up to 54/62) are described as a N-terminal poly-glutamine stretch (Poly Q) and according to several USP7 deletion experiments, have been proved to exert low influence over its enzymatic activity or binding affinity. The N-terminal domain (NTD) of USP7, in residues 54–208 where the TRAFs are located, showed the capacity to bind to p53 and Mouse double minute 2 homolog (MDM2), as well as viral proteins like the Epstein–Barr virus nuclear antigen 1 (EBNA1) and Viral Interferon Regulatory Factor 1 (vIRF1). Despite having this substrate binding action, when analysing the compared activity between a NTD-truncated USP7 variant (aa 208–1102) with a full-length USP7, it was unexpectedly demonstrated that deletion of the TRAF-like domain had no impact on USP7's activity towards MDM2 or p53, which probably results from the second MDM2/p53 binding site found recently in the C-terminal domain of USP7 (aa 801–1050 and aa 880-1050 for MDM2 and p53 binding, respectively). Regardless, TRAF-like domain has been reported to affect nuclear localization of USP7, suggesting that this domain may also have other functions not directly relevant to its enzymatic activity.[9,10,11]

The C-terminal region of USP7 is responsible for substrate recognition and binding, contributing to the development of USP7's activity by stabilizing the ubiquitin binding conformation of the catalytic domain which recognises and cleaves ubiquitin from the substrate (Fig. 2). Residues 561–1083 consist in the ubiquitin-like (HUBL) domain, while residues 1084-1102 consist in the C-terminal regulatory domain.[9,10,11]

HUBL domain has been given this denomination for sharing the grasp fold structure with the ubiquitin molecule, only lacking the terminal glycine residue essential to the

attachment of a target lysine residue. USP7 is unique for having such a vast number (5) of successive UBL domains in its C-terminal domain (aa 560–1102).[9,10,11] The HUBL domain shares the ubiquitin fold and is arranged in 2-1-2 ubiquitin units. The ubiquitin units are indicated as HUBL-1, HUBL-2, HUBL-3, HUBL-4, and HUBL-5. HUBL-1 and HUBL-2 form the HUBL-12 group, and HUBL-4 and HUBL-5 constitute the HUBL-45 group.[9,10,11] HUBL-45 is essential for USP7's catalytic domain activation and a mutation experiment indicated that deletion of HUBL-45 reduced USP7's catalytic activity. It interacts with the so-called 'switching loop' (residues 285–291) of USP7's catalytic domain and increases the activity and the ubiquitin binding affinity. Although HUBL-123 is not essential for the activation process, with the help of guanosine monophosphate-synthetase (GMPS), it can enhance USP7's catalytic activity and ubiquitin binding affinity.[9,10,11]

The C-terminal regulatory domain plays an important role in the USP7's catalytic process and its deletion would significantly diminish the enzymatic activity of USP7. It is worth noting that the proper function of the C-terminal regulator domain needs an intact HUBL-45 domain to take influence. Subsequently, analysis of the USP7 fragments function showed that deletion of the last 52 residues of USP7 C-terminus has a dramatic impact on USP7's activity, highlighting the vital role of the C-terminal domain in regulating USP7's enzymatic activity. Nevertheless, the underlying molecular mechanisms of catalytic activation are poorly understood and quite controversial. Overall, further structures of longer fragments of USP7 are still needed to provide insight into the detailed mechanism of C-terminal activation.[9,10]

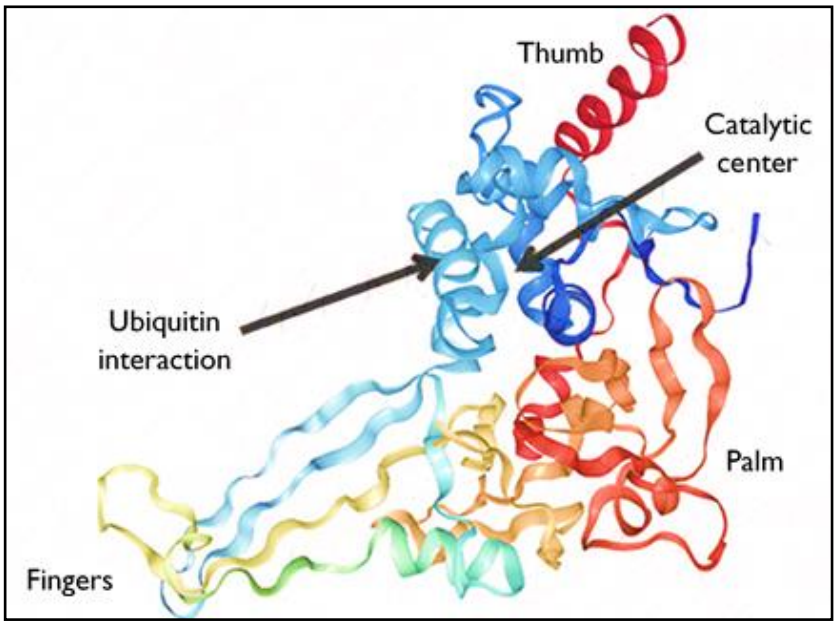


Figure 3 - The structure of USP7's catalytic domain (Protein Data Bank (PDB) identifier 1NBF). (Adapted from Yuan *et al*)[4]

The whole structure of the catalytic domain resembles an extended right hand consisting of three parts: Finger, Palm and Thumb. Deubiquitination is a multiple step process but the main concept consists in a nucleophilic attack on the carbonyl group of the isopeptide by the cysteine in the active site. More precisely, it includes Cys223, His464 and Asp481 which are recognized as the so-called catalytic triad and play an important role in the catalytic process. Asp481 is required to assist in orienting the imidazole ring of His464, which will allow His464 to deprotonate Cys223 effectively, consequently causing the release of ε-amine from the target Lys residue and producing a covalent acyl enzyme intermediate with ubiquitin. Subsequently, USP7 undergoes deacylation, with the help of water molecules, freeing ubiquitin.[9,10]

USP7 normally remains in its inactive form with a poorly aligned catalytic triad, but after ubiquitin binding, the conformation changes to the active state. In particular, the distance from SH of Cys223 to N of His464 changes from 10.17 Å to 3.60 Å. Ubiquitin makes extensive hydrogen bonds with the catalytic domain, which can be found mainly in the Finger domain with its C-terminal in the cleft between the Thumb and the Palm domains. Mutation experiments in the USP7's catalytic domain indicated that Asn218, Asn226 and Asp482 are also important for the catalytic activity and can form an oxyanion hole near the catalytic triad. In addition, His456 and Asp295 can form hydrogen bonds with Arg74 and Leu73 of ubiquitin, whilst their absence would diminish the catalytic activity.[10]

4.2. Physiological roles and oncogenic property of USP7

4.2.1. p53

Several studies have already shown the carcinogenic properties of USP7, chiefly focusing on its oncogenic property with the MDM2-p53 axis.[10,11] The dysregulation of USP7-MDM2-p53 interaction can promote the occurrence and development of tumours, therefore the design of small molecules that disrupt or prevent this interaction may be an important target for cancer therapy by regulating p53 pathway.[11]

The gene TP53 encodes the transcription factor p53 and is the most mutated tumour suppressor gene in human cancers. p53 safeguards the genome by restricting chromosomal instability through its ability to eliminate cells at risk of aberrant mitoses. The loss of p53 transcriptional activity by mutations in TP53 or activation of pathways that repress p53, are the major influencing factors to malignant transformation.[12] *In vivo* and *in vitro* studies have revealed that the loss of p53's functions both facilitates the accumulation and permits the

survival of aneuploid cells. Genomic instability fuelled by p53's loss also leads to the acquisition of additional cancer driver events with the potential to accelerate transformation, metastasis, and drug resistance.[12] The precise roles of p53 in differentiation and development have remained relatively understudied, although growing evidence suggests that, in addition to regulating cell cycle arrest and apoptosis, p53 controls 'non-canonical' programs such as autophagy, metabolism, repression of pluripotency and cellular plasticity, and ferroptosis, all of which contribute to its tumour suppressor functions.[12]

The relationship between USP7 and the p53/MDM2 complex was one of the first examples where DUBs exhibited a specific role in regulating the stability of oncogene proteins.[13] In normal cells, p53's expression levels are low, and initial response to stress-induced p53's signalling will cause a disruption for the activity of E3-ubiquitin ligases, such as MDM2. The transcription factor p53 is monoubiquitinated by MDM2, leading to its translocation, additional multiubiquitination and degradation by proteasome, losing its anticancer activity, and lowering the levels of p53. The activity of USP7 is upregulated in cancer, while the USP7 weakening leads to the accumulation of p53.[10,12]

USP7 can reverse this process by deubiquitinating MDM2 and p53, but the affinity of USP7 to MDM2 is higher than to p53. USP7 promotes the degradation of p53 indirectly by deubiquitinating MDM2, limiting the anti-cancer effects of p53.[10] On one hand, p53 binds to TRAF domain and C-terminal of USP7, and then deubiquitinates p53 directly, preventing from degradation. On the other hand, MDM2 interact with TRAF domain and C-terminal of USP7 to increase its stability by erasing the ubiquitin on MDM2, protecting it from proteasome degradation. Therefore, inhibition of the interaction between MDM2 and p53 can stabilize p53.[10,11]

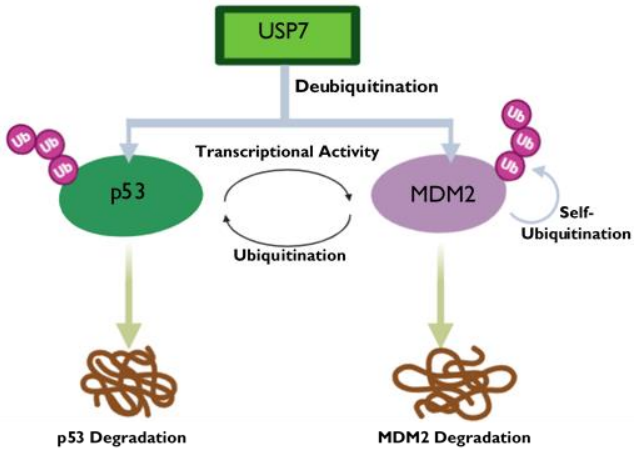


Figure 4 - USP7-p53-MDM2 axis interactions control the stability of p53 and MDM2. USP7 can stabilize p53 by deubiquitination, meanwhile, USP7 can also remove the ubiquitin of MDM2, which promotes p53 degradation. (Adapted from Wang *et al*)[11]

The p53's function is lost in about half of human cancers, making it an attractive cancer target. Identification of small molecules that allow the reactivation of p53 is also currently one of the most explored fields in cancer research. For cancers that retain wild type p53, MDM2's targeting compounds have also been described. These include small molecules such as Nutlin-3, which block the interaction of p53 with MDM2, or HLI98, responsible for targeting the ubiquitin-ligase activity of MDM2.[14] Limitations of this approach are related to the possibility of a stimulated p53's reactivation, which might cause an activation of p53 functions both in normal and in tumour cells. Optimistic observations had shown that cancers are more sensitive to p53-dependent apoptotic stimuli or senescence than normal cells, opening the possibility for the aforementioned compounds to be sufficiently selective and useful as cancer therapeutics.[14]

4.2.2. p21

In the investigation of USP7 inhibitors, p53 specific downstream genes are also being studied. In this case, the p21 roles, which are involved in a number of dysregulated pathways and the expression of the protein itself is altered in human cancers.[15] Induction of p21 in certain conditions such as DNA damage or in tumours progression depends largely on the status of the p53 protein in cancer cells, however, there are scenarios in which p21's expression pattern is independent such as in normal tissue development or cellular differentiation.[15]

In some cases, p21 might act as a genome protector and, in other cases, as the mediator of genomic instability and possibly carcinogenesis.[16] In response to p53's transcription factor activity, induction of p21 could lead to tumour growth arrest through inhibition of cyclin-kinase complex, transcription factors, and coactivators. Under different conditions, p21 can direct tumour evolution towards cancer growth through slowing down the accumulation of DNA damage, as an effort to help the cell in repairing the damage. Therefore, p21 induction has been shown to be crucial in promoting cancer cell motility and in tumorigenesis.[15]

In a p53's wildtype context, p21 and p53 act together as rescue partners of DNA damage and preserve genome stability. However, when p53 is mutated or deficient, p21 can adopt a different and 'obscure' function, promoting escape from senescence and creating cells of extreme genomic instability, which may act as cancer precursors in the tissue of the human body.[16] Although p21's mutations are rare events in cancer, it is possible that, during the evolution of a human malignancy in a mutant-p53 context, cancer cells may be at risk of acquiring additional harmful genomic changes due to a prolonged activity of p21. This

phenomenon might enhance the instability of cells and promote tumour heterogeneity, leading to new clones of cancer cells.[16] Then, whether p21 acts as oncogenic protein or as a tumour suppressor depends on its localization in the cytoplasm or in the nucleus, respectively. The controversy surrounding p21's roles in cancer evolution makes it more challenging to find the right balance in which p21's function would selectively block cancer progression, and makes it harder to have one approach for all cancer types.[15] Breakthroughs in the understanding of the diverse functions of p21 may provide valuable tools in the optimisation of treatments for p53-deficient tumours and offer a novel strategy for the development of drugs targeting p21-related pathways in these specific functional impairments.[16]

4.2.3. PTEN

A great deal of articles have suggested new correlations and roles for USP7 in the tumorigenesis process, such as its effect on the localisation of phosphatase and tensin homolog (PTEN), a dual phosphatase with both protein and lipid phosphatase activities capable of dephosphorylating phosphopeptides, as well as phospholipids, which inhibits the proliferation and migration of tumour cells.[10,11,17]

The biological effects of PTEN are dominated by its ability to dephosphorylate the lipid substrate phosphatidylinositol-3, 4, 5-triphosphate (PIP_3), converting it back into PIP_2, leading to a reduced PIP_3 production and signals. On the other hand, PI3K acts towards catalysing the reaction converting PIP_2 into PIP_3. Consequently, enzymatic function of PTEN acts as a negative regulatory signal for the mitogenic signalling and for the activation of downstream proteins of the PI3K pathway including AKT (protein kinase B), a well-known protein which plays important roles in regulating several cellular activities such as cell growth, survival, cell migration and differentiation, cell and organ size control, metabolism, etc.[17]

Mutations in certain residues of PTEN leads to an increased nuclear localization of PTEN with unknown mechanisms. Studies suggest that specific monoubiquitination regulates the carrying of PTEN between cytosol and the nucleus. Interestingly, while monoubiquitination leads to its nuclear shuttling, poly-ubiquitination of PTEN leads to its degradation.[17] In earlier findings, it was reported that PTEN would be exclusively located in the cytoplasm, but now it is clear that it can be located both in the cytoplasm and in the nucleus. Nuclear PTEN plays other roles in addition to its lipid phosphatase activity and the nuclear function of PTEN is important because it triggers the ability of PTEN to inhibit tumour development by promoting the stability and transcriptional activity of p53 by directly associating with it or by playing other important roles in chromosome stability, DNA repair and cell cycle regulation.[17]

4.2.4. FOXO

Similarly, USP7 affects tumour progression by also interacting with the activity of the Forkhead box O (FOXO) family members, including FOXO1, FOXO3, FOXO4 and FOXO6, which are transcription factors that take part in regulating several cellular responses, by affecting their activity and localization.[11] It is reported that USP7 can remove ubiquitin from FOXO1. Moreover, mono-ubiquitinated FOXO4, located in the nucleus, exhibits stronger transcriptional promotion activity. USP7 can, in turn, suppress FOXO4's activity through its deubiquitination and re-localization. In brief, USP7 affects tumour evolution by interacting with FOXOs and modelling their activity and localization.[11]

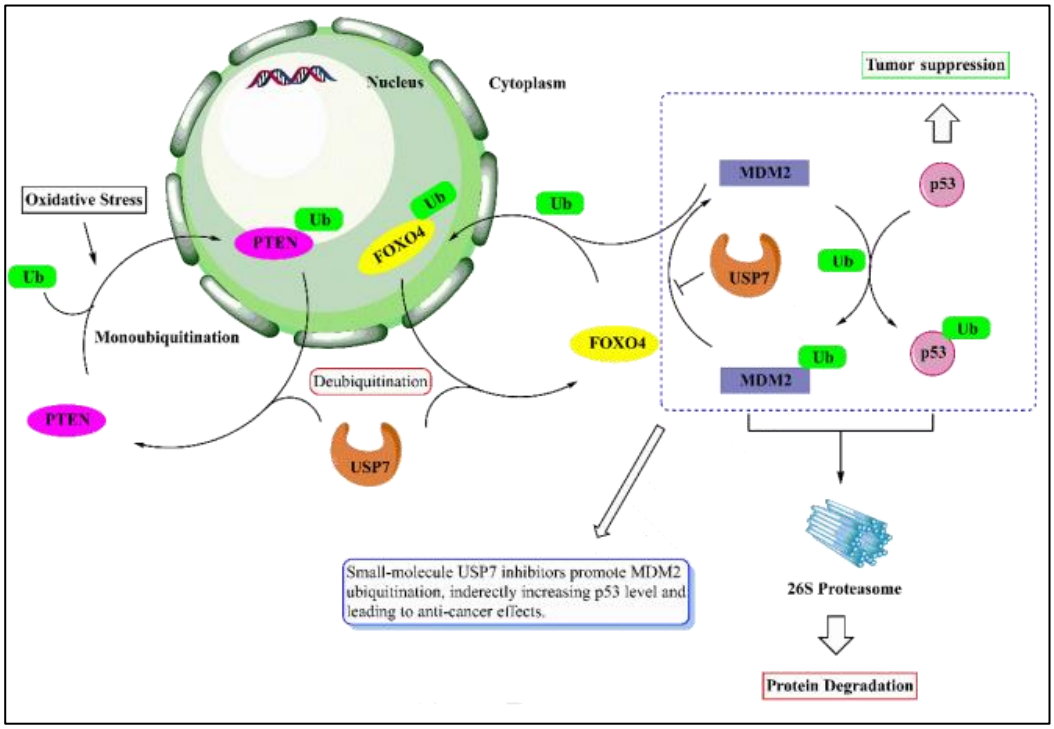


Figure 5 - Oxidative stress in cell leads to the ubiquitination and a nuclear localisation of PTEN and FOXO4, where they are able to trigger cell apoptosis and enhance transcriptional activity. However, USP7 can deubiquitinate them and lead them to the cytoplasm where they lose their anti-tumour effects. (Adapted from Li *et al.*)[10]

4.2.5 Cell Cycle Checkpoints

Human genomic DNA is threatened by a variety of agents, such as reactive oxygen species, ultraviolet radiation (UV), chemotherapeutic drugs or DNA replication stress. To preserve the integrity of genome, a DNA damage response (DDR) is initiated. This consists in an extensive network of pathways able to identify and signal DNA damage for subsequent processing. The DDR strategies comprehend two main procedures: the activation of cell cycle

checkpoints, which gives cells time to fix the damage, before stepping into the next phase of the cell cycle or allow the direct facilitation in the DNA reparation.[18]

USP7 was described as responsible for preserving the steady-state levels of claspin, a checkpoint mediator and an adaptor protein that facilitates the ATR-mediated phosphorylation and activation of Checkpoint kinase 1 (Chk1), a crucial effector kinase in DNA damage response.[18] The cell cycle checkpoint proteins ataxia-telangiectasia mutated and Rad3-related kinase (ATR) and its major downstream effector Chk1, prevent the entry of cells with damaged or incompletely replicated DNA into mitosis when the cells are confronted with DNA damaging agents, such as radiation therapy or chemotherapeutic drugs, major modalities to treat cancer.[9] In such way, USP7 contributes to the cell Cycle Control by timing the duration in checkpoint responses.[9,18]

5. USP7 Inhibitors

DUB inhibitors and agonists are required to understand disease associations. Although multiple lines of evidence indicate an important role for DUBs in disease, the aetiology of a disease development or progression in humans is generally hard to establish unless there is a traceable germline defect. Ultimately, the development of DUB inhibitors and agonists for *in vivo* studies, including clinical trials, will allow the distinction between the drivers and the by-standers of the small molecules in investigation.[19] Through academic and industry efforts, small-molecules with DUB inhibition activity have been discovered, however there are some selectivity issues, since these compounds have often shown activity in more than one DUB. In addition, the ones that appear selective sometimes are not tested against all human full-length DUBs.[19]

USP inhibitors have a particular interest as they provide an intermediate level of "selectivity," aiming for a narrower range of substrates when compared with proteasome inhibitors.[14] Even though new small molecules, like in the case of bortezomib's therapy, proved to be a major advance and were considered a success of the proteasomal inhibitors in treating multiple myeloma, it has been associated with possible off-target toxicities and the development of drug-resistance.[13,20] So, efforts still need to be made on the discovery and improvement of small molecule inhibitors on other main elements of UPS, including inhibitors of DUBs, or E3 ubiquitin ligase.[20]

The reported USP7 inhibitors detailed in this article can be classified into five types: substituted thiophene derivatives, acridine derivatives, quinazolin-4-one derivatives, indeno

[1,2-b] pyrazine derivatives and 2-amino-4-ethylpyridine derivatives.[10] Some natural products will also be addressed as interesting resources for USP7's inhibition.

These inhibitors act through two kinds of mechanisms: the development of covalent or non-covalent bonds to the receptor. The covalent inhibitors mainly target Cys223 of USP7's catalytic domain by forming covalent bonds and blocking the combination of ubiquitin with this domain. However, due to the high homology between the USPs catalytic domains, these compounds exhibit limited selectivity. The non-covalent inhibitors mainly bind to the allosteric site near the catalytic centre and show better selectivity against other DUBs.[10]

Academic and industry efforts are focused especially on the development of new types of USP7 inhibitors due to its multifunctionality in many types of diseases and currently there are no approved drugs targeting USP7 on the market. Additionally, there is an article that evaluates the existing review articles on deubiquitinase inhibitors as only providing an extensive overview of the current research status, without sufficient information to support further work.[10]

5.1 Overview of USP7 Inhibitors

5.1.1. Substituted thiophene derivatives

5.1.1.1. P5091

Biochemical and tissue culture studies showed that thiophene compounds exhibited specific and selective inhibitory activity against USP7.[13] P5091 is a selective inhibitor of USP7 and is a tri-substituted thiophene with dichlorophenylthio, nitro, and acetyl substituents mediating anti-USP7 activity. P5091 was discovered using an Ub-PLA2 (ubiquitin phospholipase A2 enzyme) reporter assay in a high throughput screening of USP7 inhibitors, from a diversity-based library of small molecules.[9,10,20]

P5091 exhibited potent, specific, and selective deubiquitylating activity against USP7 and USP47 with an EC_{50} value of 4.2 μM for USP7, demonstrating no inhibition on other DUBs or other families of cysteine proteases tested.[9,10,20] Studies made with cell lines showed that P5091 accelerates the degradation of MDM2 and inhibit the growth of HCT116 cells (a human colorectal carcinoma cell line) (wild type) depending on time and concentration and did not produce any effects on the USP7-knockdown HCT116 cells. More significantly, it was able to prolong the life expectancy and diminished the tumour weight of mice in four kinds of haematological malignancies models.[9,10,20]

Cytotoxicity was observed in MM cells at a concentration of 3 μM, an interesting result since MM cells show resistance to Bortezomib, a 1st line substance used in MM treatment.[9,10,20]

Furthermore, pre-clinical experiments also demonstrated that combining P5091 with other standard substances used in cancer treatment like lenalidomide, HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) or dexamethasone, produced a synergistic anti-MM activity.[9,10,20]

The cytotoxic activity observed with P5091's treatment seemed to not be totally dependent on p53's upregulation only. The reason why this is so was the observation of how a p53's depletion by siRNA did not affect P5091-mediated MM cells apoptosis, contrary to the lowered cytotoxic effects observed with a MDM2 inhibitor, the Nutlin-3A, in the p53-depletion cells.[13] Another reason was the P5091's treatment that showed a decrease in the viability of p53 null ARP-1 MM cells, associated with reduced MDM2 levels and increased p21 expression. These reports indicate that, although P5091 increases p53 levels, its cytotoxic activity is not solely dependent on p53.[9,20] Furthermore, it was also observed that the silencing of p21 with siRNA attenuated P5091-induced cytotoxicity. In addition, it has been established that MDM2 could directly bind to and negatively regulate p21 by facilitating its degradation. Consequently it is acceptable to predict that P5091-induced cytotoxicity is partly mediated through MDM2.[9]

The MDM2 targeting is a validated approach to promote the restoration of p53's function, however, there are some limitations for MDM2 inhibitors such as Nutlin-3. Although Nutlin-3 can block the interaction of MDM2 and p53, Nutlin-3 cannot decrease the level of MDM2 protein. In fact, it increases the expression of MDM2 mRNA through the p53-MDM2 positive feedback loop and protects MDM2 from degradation, leading to a rapid rebound of cancer cell growth upon temporary removal of the inhibitor.[13]

This hypothesis is not only according to the demonstration that the cytotoxicity of P5091 had been significantly reduced in $p53^{-/-}$/$MDM2^{-/-}$ MEFs (Mouse embryonic fibroblast) versus $p53^{-/-}$ MEFs, but also confirmed by the potency that persisted on p53 mutated/deleted CLL (Chronic Lymphocytic Leukemia) cell lines.[9,21] One explanation can be the occurrence of a possible dual inhibition of USP7 and USP47, both of which appear as oncoproteins and druggable targets. After P5901's findings, several analogues from this molecule were developed with improvements in potency, solubility, and pharmacokinetic profile, exemplified by P22077 and P50429.[9]

5.1.1.2. P22077 and P50429

P22077 and P50429 are derivatives of P5091.[22] Although these have found great responses, such molecules still have some issues like having poor solubility and producing toxicity in relatively higher doses to achieve significant efficacy *in vivo* studies.[13]

The IC_{50} values of P22077 and P50429 against full-length USP7, calculated by the Ub-PLA assay, demonstrated 8.0 μM and 0.42 μM, respectively. P5091 was used as a reference drug (IC_{50} = 4.2 μM against USP7; IC_{50} = 4.3 μM against USP47).[10] Both compounds were irreversible covalent USP7 inhibitors and could bind to catalytic Cys223 and occupy the active site of USP7, thus blocking the enzyme activity. They also exhibited USP47's inhibitory activity.[10,23]

P22077 demonstrated significant effect on neuroblastoma (NB) cells by inhibiting USP7 which resulted in the reactivation of p53, and consequently the inhibition of tumour growth. Neuroblastoma is an embryonic tumour of the sympathetic nervous system, derived from neural crest cells and is the most common extracranial solid tumour in children.[10,24] In the same study, P22077 was also able to enhance the cytotoxic effects of doxorubicin and etoposide, and sensitise the chemo-resistant LA-N-6 NB cells.[10,24] Additional cell tests indicated that both P22077 and P50429 exhibited anti-proliferation activity against HCT116 cells. Specifically, P50429 at 30 μM for different times denoted the accumulation of p53 and p21 collected in western blot data, after cell treatment. Furthermore, P50429 did not affect the activity of caspase 3, calpain 1 and 26S proteasome.[10]

Curiously, there is a study that indicates how an increased endoplasmic reticulum (ER) and oxidative stress, rather than the MDM2-p53 axis, could be more critical for cancer cell death when induced by pharmacological inhibition of USP7, via P5091 and P22077.[9,25] In this study it is stated that USP7 inhibition causes ER stress through accumulation of polyubiquitinated proteins. For this reason, ER stress caused by USP7 inhibition contributes to oxidative stress and apoptosis in cancer cells, dismissing the need for the MDM2-p53 axis, since ER stress facilitates cancer cell apoptosis by pharmacological inhibition of USP7 activity.[25] All these correlated and plausible mechanisms can reveal that p53 independent/partial function might play a bigger role in the pharmaceutical profiling of USP7's inhibition than it has been anticipated.[9]

5.1.1.3 C7 and C19 Derivatives

The C7 and C19 derivatives were discovered in a study where a scaffold hopping of P5091 and P22077 was executed. Besides these two compounds a series of thiazole derivatives from C1–C21 were designed and synthesized based on the lead compounds.[10] The most potent compounds were naturally the C7 and C19, which exhibited a good inhibition activity at low micromolar concentrations. Both of these compounds indicated and induced cell death in enzyme and cell proliferation assays regardless of the p53's status.[13]

The IC_{50} against full-length USP7, using Ub-Rho as substrate in the screening, were 0.67 μM and 1.35 μM, in C7 and C9, respectively. P22077 was used as reference drug (IC_{50} = 19.33 μM against USP7). C7 and C19 exhibited moderate anti-proliferation activity against the HCT-116 cell line, tested by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Compared to the control compound P22077 (IC_{50} = 8.82μM), C7 (IC_{50} = 8.45 μM) and C19 (IC_{50} = 6.36μM) exhibited an improved anti-proliferation activity.[10,13] The same study deepened the relation that C7 and the thiophene derivative P22077 had, and results showed a high probability that they share a similar binding site, the ubiquitin-binding pocket of the catalytic domain of USP7, and a similar structure-activity relationship.[13]

5.1.2. Acridine Derivatives

5.1.2.1. HBX19818 and HBX28258

Through the screening of a synthetic chemical library using full-length USP7 as the functional enzyme, and ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC) as the substrate, it was discovered the analogues HBX19818 and HBX28258 which exposed an irreversible covalent USP7's inhibitor activity.[10,26] These two compounds are 9-chloro derivatives of amidotetrahydroacridine, and bind directly to USP7 by forming a covalent bound with Cys223. HBX19818 and HBX28258 revealed an IC_{50} of 28.1 μM and 22.6 μM, respectively.[10,26]

HBX19818's treated cells reduced the levels of UV-induced Chk-1 phosphorylation, decreased the Claspin protein levels, increased the levels of ubiquitinated MDM2 and consequently MDM2's degradation.[9,26] HBX19818 demonstrated, compared to another USP7 inhibitors, like HBX41108, an appreciable level of selectivity towards USP7 when measured against other related USPs such as USP2, USP5, USP8 and USP20 as well as against USP7 related deubiquitinating enzyme ubiquitin C-terminal hydrolase L1 (UCH-L1) and UCH-L3. Tests in living cells showed HBX19818 decreased HCT116 cell proliferation.[9,10,26]

Deficiencies in DDR genes facilitate cellular transformation and/or tumour development, allowing mutations and chromosomal alterations to accumulate, and consequently increase genomic instability.[27] It has been analysed in a study the role of USP7 as a new therapeutic target for CLL, a tumour model with a high occurrence of ATM/TP53 aberrations, leading to the elimination of CLL cells *in vitro* and *in vivo* independently of ATM's and p53 function, which play essential roles in coordinating DNA repair with apoptosis as a DDR.[27] Accordingly, it was demonstrated that inhibition of USP7 caused selective loss of CLL viability independently of ATM and p53 function, mediated through the accumulation of

unrepaired DNA double-strand breaks. High levels of USP7's expression in primary CLL samples were also observed, and consistent with previous studies it was suggested that USP7's dysregulation could contribute to the pathogenesis of lymphoid malignancies.[27] Furthermore, a new mechanism was identified whereby USP7's inhibition initiated p53-independent killing of malignant cells. Through the collected data it was suggested that an accumulation of DNA damage in CLL cells would stimulate the production of polymeric adenosine diphosphate ribose (poly (ADP-ribose) or PAR) through the overactivation of Poly (ADP-ribose) polymerase 1 (PARP1), triggering into necrotic death possibly because of the depletion of ATP.[27] PARP1 is a crucial mediator of extra-and intracellular stress signals and the cellular resolutions depends on the intensity of its activation, being able to trigger an initiation of the DNA damage repair machinery or the beginning of cell death. In cases of persistent stress, extended activation of PARP1 can impair the energetic balance of cells, leading to dangerous ATP depletion, triggering apoptosis.[28] Along with other results, HBX19818 was highly synergistic when used in combination with the chemotherapy drugs cyclophosphamide and mitomycin C, leading to the anticipated conclusion that a combination with a USP7 inhibitor could possibly allow for lower doses of chemotherapeutic agents to be administered to frailer patients with CLL, suggesting USP7 as a promising therapeutic target for the treatment of refractory CLL.[27] The study did not test the full potential off-target effects of HBX19818 and since other inhibitors had shown activity in other DUBs, such as P5091 in relation to USP47, there is the possibility that some of the antitumor activity with HBX19818 has resulted in off-target effects.[27]

5.1.3. Indeno[1,2-b]pyrazine Derivatives

5.1.3.1. HBX41108 and HBX91490

HBX41108, is a 9-oxo-9H-indeno[1,2-b] pyrazine-2,3-dicarbonitrile derivative and a reversible USP7 inhibitor. These compounds were found through a high throughput screening against a chemical diverse library containing 65092 compounds and kinetic analysis showed that HBX41108 inhibited USP7 in an non-competitive way, indicating that it preferentially interacts with the enzyme-substrate complex rather than it directly competing with substrate binding.[10,14,29]

HBX41108 had an IC_{50} = 424 nM against full-length USP7 with Ub-AMC as substrate, however it was more potent against USP8, displaying an inhibitory activity against USP8 with an IC_{50} = 96 nM. The HBX91490, an HBX4118 analogue, still revealed more inhibitory activity against USP8, exhibiting an IC_{50} = 96 nM against USP8. Interestingly enough, this analogue with

a hydroxy group at C7 was an absolute USP7 inhibitor, with IC_{50} = 66 μM against USP7, however it did not demonstrate discernible activity against USP8 (IC_{50} >100 μM), having some drug potential for future research.[10] Cell tests indicated that the HCT116 cell line was more sensitive to HBX41108 with an IC_{50} = 0.27 μM than normal diploid NIH-3T3 fibroblasts with an IC_{50} = 1.77 μM. However, a concentration of 3 μM did not affect the viability of normal human hepatocytes.[10,14,29]

A study was conducted on human adipose-derived stem cells (hASCs), multipotent stem cells with self-renewal capabilities and differentiation potential, including osteogenesis, where the objective was to evaluate and characterize the effects of USP7 on osteogenic differentiation of hASCs. To this end, experiments *in vitro* and *in vivo* were carried out through genetic depletion or overexpression of USP7. Then It was revealed that HBX41108 facilitated the repression of osteogenic genes, and consequently inhibited osteogenic differentiation of hASCs.[30] Furthermore, in cell viability and apoptosis assays, with HBX41108 different doses, it was revealed that concentrations of less than or equal to 1 μM had a small influence on cell proliferation and apoptosis.[30] It was then suggested that USP7 inhibitors may have therapeutic potential for hyperplasia of bone formation and since HBX41108 has been reported as a potential anticancer drug, the findings in this study can possibly anticipate that USP7 inhibitors may harm the osteogenic ability and cause related side effects such as osteoporosis during antineoplastic therapy. However conclusive evidence of the correlation between the USP7 inhibitors and bone homeostasis and bone development, has not yet been reported, and awaits further investigation.[30]

Another research article studied the influence of USP7's deubiquitinating activity on PPARγ (peroxisome proliferator-activated receptor γ) stability, having demonstrated that treatment with HBX41108 and consequent inhibition of USP7 in cell lines could decrease the transcriptional activity of endogenous PPARγ.[31] PPARγ is a member of the nuclear hormone receptor superfamily and a ligand-activated transcription factor which plays a significant role in a multiplicity of physiological processes, such as adipogenesis, glucose homeostasis, lipid metabolism, and osteogenesis. PPARγ is polyubiquitinated in response to ligand binding or phosphorylation and then degraded by the proteasome.[31] HBX41108 was able to significantly reduce the basal transcriptional activity of PPARγ by up to 70% and eradicated the induced transcriptional activity, further validating that USP7 stabilizes PPARγ.[31]

5.1.4. 2-Amino-4-ethylpyridine Derivatives

5.1.4.1. GNE6640 and GNE6776

GNE6640 and GNE6776 are also non-covalent reversible USP7 inhibitors and were discovered by combining NMR-based (nuclear magnetic resonance) screening of structure-based with drug design strategy, while the Ub-Rho110 assay validated the screen results. The binding modes of these two compounds are distinct from the previously described compounds, bonding to an allosteric site at a 12 Å distance from the catalytic Cys223.[10,32,33]

GNE6640 had an IC_{50} = 0.75 μM against full-length USP7, while GNE6776 had IC_{50} = 1.34 μM, using Ub-Rho110 as the substrate. Both compounds exhibited good selectivity against 36 other DUBs. GNE6776 remained selective even at 100 μM, a sixfold higher concentration than used in cellular assays.[33] GNE6640 could decrease the viability of 54 cell lines with $IC_{50} \leq$ 10 μM, while GNE6776 decreased the viability of 6 cell lines with an $IC_{50} \leq 10$ μM.[10,32,33]

GNE6640 was combined with doxorubicin, cisplatin and other DNA damaging agents to achieve an activation of p53's response and produced an enhanced USP7's inhibitory efficacy. Through this result, a strategy to identify new pathways USP7-related employing the inhibition activity of GNE6640 and GNE6776, in combination with DNA damaging agents was formulated. So, by the screening of acute myeloid leukaemia (AML) cell line with GNE6640 or GNE6776 in combination with 589 chemotherapeutic and related targeted agents some interesting outcomes were revealed, especially with PI3K and PIM kinase inhibitors, disclosing a new connection between USP7 deubiquitinase activity and PIM kinases in regulating the cell viability.[33]

In combination, GNE6776 and GDC0570 (PIM inhibitor) treatments increased the cleavage of PARP and caspase-3, mechanistically confirming the cell viability studies.[33] GNE6776 also displayed a good oral bioavailability and half-life in mice, although achieving target plasma exposure only momentarily, due to its high plasma protein binding and lack of submicromolar cellular EC_{50}. Therefore, improving both these parameters will enhance the ability of these series to achieve effective concentrations *in vivo*. The study concluded that a profiling of the aforementioned series both *in vitro* and *in vivo* toxicity assays would be essential to identify possible negative side effects.[32,33]

5.1.5. Quinazolin-4-one Derivatives

5.1.5.1. XL188 and XL203C

XL188 and XL203C are derivatives of quinazolin-4-one bearing a tertiary hydroxypiperidine moiety. XL188 displayed an excellent USP7's inhibitory activity with IC_{50} =

193 nM against the USP7's catalytic domain and 90 nM against full-length USP7, using Ub-AMC as the substrate. In contrast, its enantiomer, XL203C, was almost eighty-fold less potent than XL188 with an IC_{50} value of 10.7 μM against the USP7's catalytic domain and 7.18 μM against full-length USP7.[10,34] Both analogues are non-covalent selective USP7 inhibitors and the inhibition of the USP7 active site was achieved by blocking the combination of ubiquitin with the USP7's catalytic domain. Additionally, XL188 and XL203C were sufficiently selective against other DUBs at 10 μM.[10,34]

In MCF7 cells (a breast cancer cell line) treated with XL188, levels of p53 and p21 increased following 16 h of treatment with concentrations in line with the IC_{50} of native USP7. Consistent with the effects being USP7 dependent, no observable changes in the same proteins were observed under similar conditions and across the same range of concentrations of XL203C, the inactive enantiomer,[34] Other data demonstrated the degradation of MDM2 by XL188 was also observed in the same system where new protein synthesis was obstructed by addition of cycloheximide. The need to block protein synthesis to observe degradation of MDM2 was consistent with the established negative feedback loop affecting these proteins in which p53 transcriptionally upregulates MDM2. Comparably, treatment of multiple myeloma MM.1S cells with XL188, but not XL203C, led to loss of MDM2 supplemented by an increase in downstream tumour suppressors p21 and p53, as has been previously reported for the USP7 inhibitor P5091. XL188 is described as an inhibitor of USP7 with a double-digit nanomolar potency towards USP7 and excellent selectivity when compared to the largest available panel of purified DUBs.[34]

5.1.5.2. Compounds 4,6 and 46

Surface plasmon resonance (SPR) screening of 1946 fragments indicated that the fragment thieno[2,3-d]pyrimidin-4-one had high affinity USP7's catalytic domain. Through additional combinations of this moiety and based on previously reported USP7 inhibitors, the discovery of an initial compound (compound 1) was materialized, with an IC_{50} = 15.1 μM against full-length USP7, using Ub-Rho110 as the substrate.[10,35,36] Subsequently, through the development of this first compound, recurring to identification via fragment-based screening, scaffold-hopping, hybridization and optimization exercises, correlated compounds were synthesized.[35,36] Additional profiling revealed that compound 1 had excellent aqueous kinetic solubility (> 200 μM) and was free from redox cycling activity, a liability that has the potential to lead to false positive readouts in biochemical assays.[36] The cocrystal structure of compound

2, an analogue of compound 1, with the USP7's catalytic domain indicated an allosteric binding site about 5 Å away from the Cys223. The basic interactions were similar to analogue XL188.[10]

Compound 4, the most potent USP7 inhibitor recorded in the research against full-length USP7 (IC_{50} = 1.5 nM), emerged from the cocrystal data and from a structure-based drug design strategy.[10,35] It exhibited high solubility, chemical stability and was also free of redox cycling activity. The high specificity of this compound for USP7 was recognized in cells, as supported by the lack of engagement against USP47, the closest USP7 homolog. Under similar conditions, it was highly potent against USP7 in cellular target engagement assays. Its enantiomer (compound 6) was, however, less potent with IC_{50} = 1.9 μM.[35]

Analysis of molecular structures of compound 4 revealed also a binding outside of the active site in a previously undisclosed allosteric pocket situated 5.5 Å from the catalytic cysteine.[35] Suggested mechanisms proposed an action in preventing the alignment of the catalytic triad within the active site of USP7, and additionally hint at an induced local distortion of the Ub tail, preventing catalysis. It was clarified that these mechanisms are not mutually exclusive and can act collectively, being consistent with the non-competitive mode of inhibition demonstrated.[35]

Concentration-dependent pathway modulation was subsequently demonstrated, leading to increased protein levels of p53 and reduced levels of the oncoprotein MDM2, as a concomitant induction in p21's protein levels was also observed. The observations were extended to multiple cell lines and are consistent with the expected mechanism of a USP7 inhibitor.[35] The anti-proliferative effects of these compounds were later tested, which led to the identification of hypersensitive cell lines to USP7 inhibition (EC_{50} < 30 nM), including both haematological (RS4-11) and solid tumour cell lines (LNCaP). Apoptotic induction was observed in RS4-11 cells, as evidenced by the PARP's and caspase-3 cleavage. Furthermore, compound 4 demonstrated more potent anti-proliferative activity against established MDM2 antagonists.[35]

Moreover, although the monocyclic compound 46 exhibited excellent USP7's activity with IC_{50} = 0.09 μM, it was less potent than compound 4.[10,36] Besides that, further exploration on compound 46 showed excellent selectivity versus a panel of 21 USPs when screened at a fixed concentration of 10 μM, as well as potent intracellular USP7 target engagement (EC_{50} = 0.32 μM) in cells. The aqueous kinetic solubility of compound 46 was high (Ksol >190 μM), but the Caco-2 A/B permeability assay at pH 6.5 was found to be low (Papp < 0.3 × 10−6 cm/s), limiting their potential for oral dosing.[36]

The pharmacokinetic profiles of compound 46 were assessed in male CD-1 mice and, as expected from their low Caco-2 permeabilities, exhibited poor oral bioavailability (F < 1%),

however, when dosed intraperitoneally, a reasonable bioavailability was exhibited (F = 44%). Additionally, compound 46 demonstrated low plasma clearance (CL= 13 mL/min/kg) and a low volume of distribution (Vss ≤1 L/kg). Researchers are looking to improving pharmacokinetic profiles based on the preliminary *in vivo* results.[36]

5.1.5.3. FT671 and FT827

A screening of 50,000 diverse compounds by the Ub-Rho assay and further confirmation by SPR led to the discovery of a hit compound containing the pyrazolo[3,4-d]pyrimidin-4-one moiety, while further optimisation resulted in the analogues FT671 and FT827.[10,37]

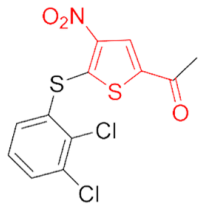
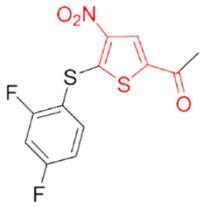
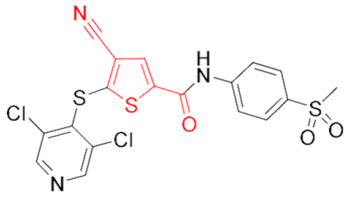
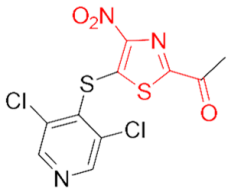
FT671 was a non-covalent selective USP7 inhibitor with IC_{50}= 52 nM against the USP7 catalytic domain and 69 nM against full-length USP7 using Ub-Rho as the substrate. Instead, FT827 was a covalent selective USP7 inhibitor with a value of 66 ± 25 $M^{-1}s^{-1}$ in the K_{inact}/k_i assay, originated for SPR measurements, against full-length USP7.[10,37] Both compounds bound to the inactive USP7 apo-state and competitively blocked the binding of ubiquitin. They also exhibited good selectivity against 38 DUBs from diverse families, but no activity against USP47 and USP10, two enzymes inhibited by P5091/P22077.[10,37]

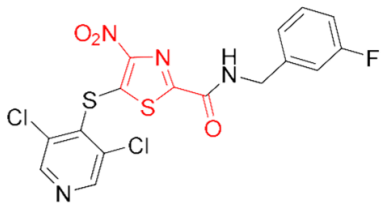
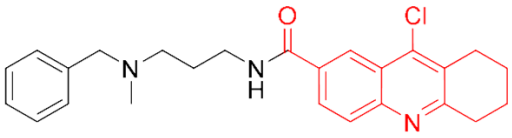
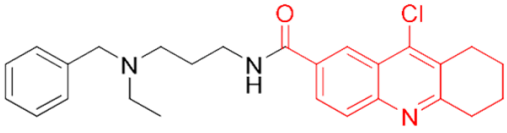
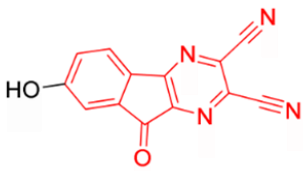
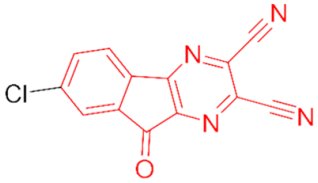
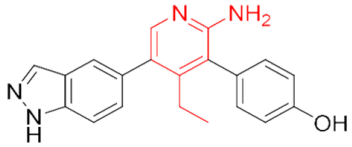
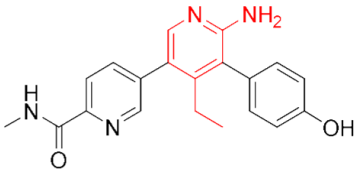
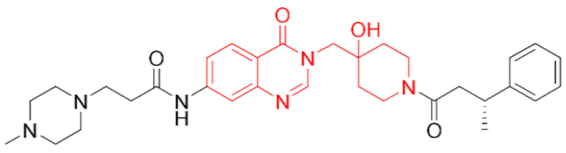
Biological activity research on FT671 noticed the increase of p53's protein levels in cell lines such as HCT116 or U2OS (human tissue of bone with osteosarcoma), inducting p53's target genes. More data showed the degradation of N-Myc and upregulation of p53 in the neuroblastoma cell line IMR-32 caused by FT671.[37] N-Myc is a transcription factor related with neuroblastoma tumorigenesis and its activity is, usually, strictly controlled by ubiquitination dependent proteasome degradation. USP7's expression is more ample in patients with neuroblastoma who have a worse prognosis, having correlated expression with N-Myc transcriptional activity.[38] The same report additionally stated that FT671 incited the degradation of proteins, which USP7 also regulates, such as UHRF, an E3 ligase and DNMT134,35, a DNA methylase, confirming its influence in USP7 activity.[37]

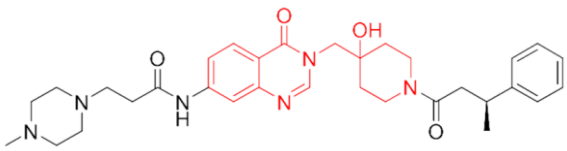
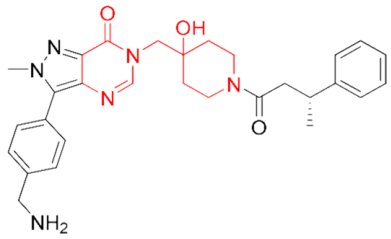
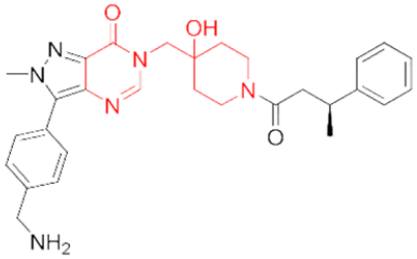
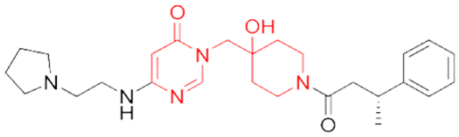
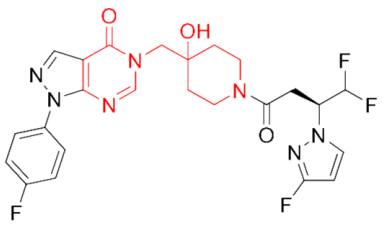
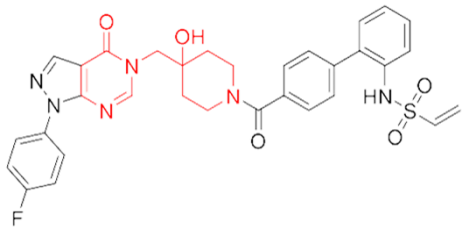
As for other cell lines, FT671 stabilised p53 in MM.1S cell line, which correlated with an increased MDM2's ubiquitination and expression of p53's target genes. FT671 blocked the proliferation of MM.1S cells with an IC_{50} of 33 nM.[37] Experiments on the effects of FT671 on the p53-MDM2 axis *in vivo*, utilized a MM.1S xenograft mouse model in which non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice which were implanted with MM.1S cells and randomized into different treatment groups.[37] For pharmacodynamics studies, the analysed inhibitor was administered once-a-day at 25 $mg.kg^{-1}$, 75 $mg.kg^{-1}$ or 200 $mg.kg^{-1}$ via

oral gavage. Individually, the mice were sacrificed at different time points, and compound effects on p53 were monitored.[37] Reflecting the studies in cell lines, p53's stabilisation in tumour tissue was observed shortly after a single dose of FT671. After 24 h, p53 levels returned to those of the vehicle treated control.[37] In addition, *in vivo* anti-tumour growth activity of FT671 was tested in the MM.1S xenograft model after daily dosing via oral gavage at 100 $mg.kg^{-1}$ and 200 $mg.kg^{-1}$, leading to a significant dose-dependent tumour growth inhibition. FT671 was well tolerated even at high doses, and no significant weight loss or cachexia was observed during the study.[37]

Table 1 - Summary of IC_{50} for USP7 and molecular structure of representative USP7 inhibitors. (Adapted from Li *et al.*)[10]

	Name	Structure [(a)]	IC_{50} for USP7 [μM]	Binding mode	References
Substituted thiophene derivatives	P5091		4.2 (EC_{50})	Covalent	[20]
	P22077		8.0	Covalent	[22; 23]
	P50429		0.42	Covalent	[22; 23]
	C7		0.67	Covalent	[13]

	C19		1.35	Covalent	[13]
Acridine Derivatives	HBX 19818		28.1	Covalent	[26]
	HBX 28258		22.6	Covalent	[26]
Indeno[1,2-b]pyrazine Derivatives	HBX91 490		Inative	Non-Covalent	[14; 29]
	HBX41 108		0.424	Non-Covalent	[14; 29]
2-Amino-4-ethylpyridine Derivatives)	GNE66 40		0.75	Non-Covalent	[32; 33]
	GNE67 76		1.34	Non-Covalent	[32; 33]
Quinazolin-4-one Derivatives	XL188		0.090	Non-Covalent	[34]

	XL203 C		7.18	Non-Covalent	[34]
	Compounds 4		0.0015	Non-Covalent	[35]
	Compounds 6		1.9	Non-Covalent	[35]
	Compounds 46		0.09	Non-Covalent	[36]
	FT671		0.052	Non-Covalent	[37]
	FT827		0.065[(b)]	Non-Covalent	[37]
a) The important mother cores for the activity are coloured red. b) The K_{inact} /K_i values obtained from SPR measurements.					

5.2. Natural Products

Recent efforts in the areas of natural product's isolation, synthesis and screening have acknowledged a diversity of USP7's inhibitory compounds derived from natural sources. Spongiacidin C, a pyrrole alkaloid, obtained from screening the extracts of 700 marine

invertebrates and isolated from the marine sponge *Stylissa massa*, displayed a heightened antagonist activity against full-length USP7 with an IC_{50} value of 3.8 μM, and specificity to USP7 and USP21. This finding shed some light on new structural possibilities to promote the inhibition of USP7, although its effect on USP7's activity in cells remains a mistery.[6,10] Other examples of natural products are the Ursolic acid, a natural pentacyclic triterpene with moderate USP7's inhibitory activity (7.0 μM). Cell tests indicated that this compound could inhibit the proliferation of myeloma cells with IC_{50} = 6.56 μM in a dose-dependent manner, and the Xestoquinone, a pentacyclic quinone isolated from the marine sponge *Petrosia alfiani*, identified as a potent USP7 inhibitor which exhibited an IC_{50} = 0.13 μM.[10] Besides these three examples, there are other natural compounds reported like Sulawesin A or an identified synthetic triterpenoid, also known as bardoxolone methyl.[6,10]

6. Conclusion

DUBs are a class of emerging antineoplastic targets for their sparing functions such as preventing substrate proteins from being degraded in the proteasome, and their abilities to modulate protein fate in a specific manner. Specifically, when the target protein is an oncogenic protein, the related DUB could enhance its stability and thus promote carcinogenesis or tumour progression, which supports the identification of inhibitors against deubiquitinases as promising therapeutic agents.[9] DUBs play vital roles in these processes by having responsibility in control cell identity and self-renewal, implying that they have potential to be pertinent drug targets.[10,19] In support of this hypothesis, all the disease associated with DUBs, and more specifically USP7, here discussed appear to have a role in regulating the status and/or the differentiation of select cells.[19] The irregular expression triggers several diseases, including cancer, mental disorders, and immune diseases. In particular, USP7 and its involvement in multiple pathways has been thoroughly investigated and is becoming further well understood, showing signs of being a rather promising target for cancer therapy.[10] Although the capacity to control cell identity requires and involves proven clinical benefits on the therapy for certain pathologies, a greater understanding of DUBs in normal physiology is still necessary to avoid harmful side effects.[19]

The development of potent and selective small molecules with USP7's inhibitory activity still has a long road ahead and there is an urgency for greater efforts to be made in order to accelerate the development process, allowing us to make sense of pre-clinical studies and benefit patients in dire need; remembering that, currently, there are no approved drug targeting USP7 available on the market.[10]

Despite the variety of the already described USP7 inhibitors, there are several matters of concern. The inhibitors described displayed an optimistic micromolar potency but only some of them are in the nanomolar levels, however the poor selectivity against USP7 and the cross effects related to other DUBs still exist and cannot be ignored.[10] Achieving specificity of USP7 inhibitors might be the real challenge both at the level of identifying inhibitors specific to USP7 and the level of promiscuity of USP7 toward ubiquitinated targets. The inhibitors in study have also shown p53-independent cellular responses, demonstrating that USP7's additional functions beyond p53 might also play a pivotal role in the biological response to USP7's inhibition. Nevertheless, it could not be ruled out that there were off-target effects for the known USP7 inhibitors.[9] Furthermore, the physical properties do not meet the standards for additional pharmacological evaluations. The evaluation levels of the disclosed compounds are limited to cell tests and few of them have been evaluated *in vivo* due to their poor solubility and metabolism properties.[10]

In comparison with reversible inhibitors, selective covalent irreversible USP7 inhibitors present potential advantages. For example, a comparatively short duration of treatment could result in sustained USP7's inhibition and in the prolongation of biological effects, leading to improved therapeutic efficacy.[6] In addition, development of covalent USP7 inhibitors may be governed by pharmacokinetics parameters that differ from those of reversible inhibitors, particularly with respect to half-life and clearance. Given the tissue-wide expression and functional importance of USP7 in normal cells, selective irreversible inhibitors with fast metabolic clearance could help mitigate potential on-target as well as off-target toxicities.[6] It should be kept in mind, however, that potential immunogenicity of protein adducts resulting from covalent inhibitors could generate allergic responses or drug hypersensitivity reactions.[6]

Apart from efficacy, safety is a main concern for therapy based on USP7's inhibition. Genetic deletion of USP7 leads to early embryonic death in mice, implying that USP7 is necessary in mouse development, at least in the embryonic stage. Additionally, USP7 has also shown an essential role in DNA replication and other fundamental cellular processes necessary for normal homeostasis, raising concerns about potential toxicity of USP7 inhibitors for cancer treatment.[9] Although some *in vivo* studies are quite encouraging and, despite being relatively limited, they may also advocate for the safety of a pharmacological USP7's inhibition after the embryonic stage. Undeniably, more *in vivo* data, as well as additional analysis of USP7's genetic deletion effects, are required to evaluate the safety of targeting USP7 with small-molecule inhibitors.[9]

Due to the homology of catalytic domains among USPs, research still needs to keep its focus on the structure-biology aspect and uncover new allosteric binding sites to design novel

compounds as an alternate way to improve the selectivity and the potency.[10] Improved selectivity assays will be essential in this regard to fully comprehend compound activity and enhance desired compound properties. In the cases where DUB function needs to be re-established or compensated for, it will have to be developed molecules with focus on the causes of dysregulation or on the affected pathological pathway(s) of an inactivated DUB.[19] The limited screening methods significantly affect the accuracy of the evaluation results and all the current studies employ more than one method to evaluate the compounds.[10] Methods such as Ub-AMC and Ub-Rho assays, are commonly used and cost-effective, however, if the compounds tested are fluorescent, it can cause inaccurate results. On the other hand, modern methods, such as SPR, may overcome this disadvantage, but its application to large scale compound screening is still impractical because of the high instrumentation cost and its low efficiency. A new screening method that carefully balances accuracy and efficiency would undoubtedly promote the developing process of USP7 inhibitors.[10]

In the case of some protumorigenic proteins, overexpression of USP7 in a tumour establishes a dependence of its presence for the tumour's growth and, therefore, an increased susceptibility of the tumour to USP7 inhibitors. A case in point is MM, which is described by a remarkable level of USP7 overexpression compared to normal cells and exquisite sensitivity to USP7 inhibitor treatment. This expression in tumours could serve as a predictive biomarker in future clinical studies. The observation that USP7 inhibitor synergizes with approved cytotoxic chemotherapy agents *in vitro,* in addition to overcoming resistance against bortezomib, launches opportunities for exploring the *in vivo* efficacy of USP7 inhibitors in combination with chemotherapy agents such as radiation, DNA damaging agents, kinase inhibitors, or epigenetic modulators which are expected to produce better therapeutical outcomes.[6]

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