Spirooxadiazoline Oxindoles As Potential Anticancer Agents

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**Poster**

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<th>Definition</th>
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<tr>
<td>ADME</td>
<td>Absorption, Distribution, Metabolism and Excretion</td>
</tr>
<tr>
<td>Bn</td>
<td>benzy</td>
</tr>
<tr>
<td>c</td>
<td>concentration</td>
</tr>
<tr>
<td>CDCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>deuterated chloroform</td>
</tr>
<tr>
<td>CV</td>
<td>cell viability</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
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<td>dd</td>
<td>double doublet</td>
</tr>
<tr>
<td>dt</td>
<td>double triplet</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethylsulfide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>equiv.</td>
<td>equivalent</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl</td>
</tr>
<tr>
<td>FC</td>
<td>flash chromatography</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>MDM2</td>
<td>mouse double minute 2 homolog</td>
</tr>
<tr>
<td>MDMX</td>
<td>mouse double minute 4 homolog</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>milliLiter</td>
</tr>
<tr>
<td>mmol</td>
<td>millimole</td>
</tr>
<tr>
<td>mp</td>
<td>melting point</td>
</tr>
<tr>
<td>Mut</td>
<td>Mutated</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MTS</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)</td>
</tr>
<tr>
<td>nM</td>
<td>nanoMolar</td>
</tr>
<tr>
<td>NCS</td>
<td>N-Chlorosuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NO</td>
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<tr>
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</tr>
<tr>
<td>PLK4</td>
<td>Polo-like kinase 4</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>p53</td>
<td>tumor protein p53</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>Wt</td>
<td>wild-type</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
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<tr>
<td>µM</td>
<td>microMolar</td>
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Abstract

Cancer is a large group of diseases which arises from one single cell, grows uncontrolled and may spread to other organs \(^1\). According to WHO Cancer Report (2015), it is one of the leading causes of morbidity and mortality in the modern world with 8.2 million deaths in 2012 and 22 million expected cases within the next 2 decades \(^2\). Latest statistics show that colon cancer is globally the third most commonly diagnosed cancer type and specifically the only cancer observed with approximately equal frequency in men and women \(^3\). Cancer is globally an important area of therapeutic interest. The high number of deaths is a crucial reason for science to develop new approaches and novel compounds for its treatment.

The non-selectivity and acute toxicity of many antitumor agents has prompted the search for new antitumor agents with improved tumor selectivity, efficiency and safety. Herein, our latest results on the synthesis of a novel chemical family of spirooxindoles containing an 1,3,4-oxadiazoline five-membered ring will be presented. These two scaffolds (spirooxindole and oxadiazole) are separately reported to have \textit{in vitro} antitumor activity as p53/MDM2 inhibitor \(^4,5\). However, oxindole joined with 1,3,4-oxadiazoline ring in the same molecule was never studied before as anti-tumor agents. The synthesis was performed by 1,3-dipolar cycloaddition between derivatives of indoline-2,3-diones and hydrazonyl chlorides. Using this method, twelve compounds were obtained with high yields. Additionally, all compounds were tested \textit{in vitro} in three different colorectal cancer cell lines: an isogenic matched pair of wild type p53 and deleted human colorectal cancer cell lines [HCT116 p53\(^{+/+}\) and p53\(^{+-}\)], and a human colorectal adenocarcinoma cell line [SW620 (mut p53)], in order to report cell growth inhibitory activity. From the library of spirooxadiazoline oxindoles, quite remarkably, IC\(_{50}\) values of four compounds were calculated. Three compounds displayed IC\(_{50}\) values below 10 µM and antiproliferative activity in SW620 cell line. Importantly, one compound significantly decreased SW620 cell viability, while it did not decrease normal fibroblasts viability. This indicates that compound 32a associated cytotoxicity is specific for tumor cells. In sum, spirooxadiazole oxindoles may represent a promising scaffold for the development of new antitumor agents. Ongoing studies are directed to ascertain their molecular mechanism of action and cellular toxicity, in order to confirm their potential as antitumor agents.
Keywords: Spirooxindoles; spirooxadiazoline; 1,3-dipolar cycloaddition; colorectal cancer; biological activity
Resumo

O cancro é um grande grupo de doenças que surge a partir de uma única célula, cresce descontrolado e pode se espalhar para outros órgãos. De acordo com o Relatório da OMS (2015), o cancro é uma das principais causas de morbidade e mortalidade no mundo moderno, com 8.2 milhões de mortes em 2012 e 22 milhões de casos esperados nas próximas 2 décadas. As últimas estatísticas mostram que o cancro do cólon é o terceiro tipo de cancro mais diagnosticado e único cancro observado com aproximadamente igual frequência em homens e mulheres. O elevado número de mortes por cancro torna prioritária, na área de investigação na saúde, a descoberta de novas famílias de compostos com atividade anticancerígena.

A falta de selectividade, assim como a toxicidade aguda de muitos agentes anticancerígenos, levou à necessidade de desenvolver novos agentes antitumorais com uma melhor selectividade tumoral, eficiência e segurança. Nesta dissertação, são apresentados resultados obtidos no desenho e síntese de uma nova família de espirooxindoles contendo um anel de cinco membros de oxadiazolino como potenciais agentes antitumorais. Os compostos finais resultam da junção de duas estruturas químicas (espirooxindole e oxadiazole) na mesma moléculas, que isoladamente estão descritas como tendo actividade antitumoral. A síntese foi realizada por cicloadição 1,3-dipolar entre derivados de indole-2,3-dionas e dipolos-1,3 (gerados in situ a partir de cloretos de hidrazonilo). Doze compostos finais foram obtidos com rendimentos elevados, os quais foram testados in vitro em três linhas celulares de cancro colo-rectal: um par de linhas celulares de cancro colo-rectal humano isogénicas, com e sem p53 selvagem [HCT116 p53(+/+)] e p53[−/−], e uma linha celular de adenocarcinoma colo-rectal humano [SW620 (mut p53)]. Três compostos apresentaram valores de IC_{50} abaixo de 10 µM na linha celular SW620. Em particular, foi obtido um composto promissor para o desenvolvimento de novos agentes anticancerígenos, que diminuiu significativamente a viabilidade das células SW620, não tendo apresentado citotoxicidade em fibroblastos de colon humano normal, para as concentrações testadas.

**Palavras-chave:** Spirooxindoles; spirooxadiazoline; cicloadição 1,3-dipolar; cancro colo-rectal; actividade biológica
Chapter I - INTRODUCTION
1 Introduction

1.1 Cancer

Cancer is defined by World Health Organization (WHO) as a large group of diseases (malignant tumors) which affects any part of the body as a result of personal genetic factors and external agents such as; physical, chemical and biological carcinogens. When the normal cells mutate into tumor (abnormal) cells, they split rapidly and grow uncontrollably in a multistage process. They can even spread to other organs through the lymphatic system or bloodstream which is called a metastasis.

WHO reported, from 184 countries worldwide, that approximately 14 million new cases and 8.2 million deaths happened in 2012 because of cancer. Moreover, in economically developing countries, cancer was reported with increasing numbers due to changes in lifestyle. According to GLOBOCAN 2012 results, 64% of the cancer deaths occurred in economically developed countries. The main risk factors are smoking, consumption of saturated fat, highly refined foods, sedentary lifestyles and daily stress as a result of the urbanization and modernization. Until now, several achievements against cancer were gained and the mortality was reduced with the improvements of early detection, screening and treatment in specific cancers. Nonetheless, cancer is still one of the major threat to the world health.

Cancer is the result of lack of apoptosis which causes malignant cells to keep growing without dying. Apoptosis and cell cycle arrest are processes in organisms in which DNA is under threat of damage. The period of cell cycle arrest has the opportunity to repair the damaged DNA, however, apoptosis results in direct cell death.

A transcription factor, p53, is the regulatory key tumor suppressor protein that chooses to carry apoptosis in the cells with damaged DNA. It was discovered over 35 years ago and is generally considered as “guardian of the genome”. It has several roles on the regulation of biological processes such as DNA repair, cell survival, cell death, proliferation, growth, senescence, differentiation, motility and metabolism. It is known that uncontrolled cell proliferation is the most important step for tumor cells development. Widely in all cancer types, p53 can lose its function either by direct or
indirect ways. This can be caused by oxidative stress, DNA damage, nutrient deprivation, replicative stress, etc. In these cases, p53 modifications induce p53 activation in the cell. If the level of stress is low, p53 promotes cell cycle arrest to repair its genome. On the other hand, if damage is on too serious levels, p53 starts apoptosis or senescence leading to cell death (Fig 1.1) 17.

![Diagram of p53 activating signals and responses](image)

**Figure 1.1** - The view of p53 activating signals and responses (adapted from18).

Once the cancer cells find out the inhibition of p53 controlled pathways, they suppress the regulation of apoptosis and sustain the cell cycle progression 14. The mutations in p53 protein and the decreased expression of caspases breaks the balance between cell division and cell death 19. Studies indicate that among all human tumors, approximately half have a mutated p53 gene 15. Therefore, there is no surprise that p53 protein is one of the most studied target for cancer studies 20.
According to the statistics, globally around 22 million cancer patients were identified who had defects in p53 signaling. Considering that almost 50% of them keep the mutant p53, without tumor suppressor function, the rest will retain a wild-type p53\textsuperscript{21}. Recently, Joerger et al. repaired the function of p53 tumor suppressor in mutant p53 cancer cell lines with chemical chaperons analyzing the structural and functional effects of p53 cancer mutations\textsuperscript{22}.

Colorectal cancer is the fourth leading cause of death but also the third most commonly diagnosed cancer in the world based on the recent statistics\textsuperscript{23}. According to this statistics, the number of incidence for men is higher than for women and the rates are highest in Australia/New Zealand, Europe and Northern America (Fig 1.2)\textsuperscript{11}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure12.png}
\caption{Global colon cancer statistics (adapted from\textsuperscript{24}).}
\end{figure}

Moreover, the latest statistics of cancer incidence by GLOBOCAN (2012) show that in the European Union colorectal cancer is the second most common cancer and death cause with number of victims 447.000 and 215.000 respectively. Among the countries in Europe, Slovakia, Hungary, Denmark and Croatia have the highest incidence and mortality numbers from colorectal cancer for both sexes\textsuperscript{24}.

According to the statistics, estimated numbers for colon cancer in 2030 will increase by 60% to more than 2.2 million new cases and 1.1 million deaths\textsuperscript{25}.
Moreover, incidence rates show that nearly every 1 in 20 Americans have a possibility to be diagnosed with colon cancer in their lifetime. Nevertheless, researches proved that colon cancer develops slowly between the period of time from 10 to 20 years. As any other cancer types, colon cancer also reveals resistance against drugs and therapies. With the objective of finding novel molecules to target this tumor, in this thesis, all biologic experiments were carried out using colon cancer cells.

1.2 Oxindole scaffold

The bicyclic oxindole scaffold with one carbonyl (-C=O) group and one amino (NH) group has high importance in medicinal chemistry. Among several combinations, studies indicated that the most pharmacologically active compound has substituents at the 3rd and 5th positions. Additionally, the anti-cancer potential is higher, if substituents on these positions are bulky structures.

![Figure 1.3 - Isatin and oxindole structures.](image)

Isatin was discovered in 1841, by Erdman and Laurent, and named as 1H-indole-2,3-dione. Product was obtained from the oxidation of indigo by nitric and chromic acids (Fig 1.3). Isatin can be found in a large variety of heterocyclic compounds, such as indolines and quinolones, or can be used as precursor for drug synthesis being synthetically versatile substrates. During the last decades, isatin derivatives have been reported with high biological activities as being a part of tuberculostatic and antitumor agents and substances with anti-HIV, antimicrobial, anti-inflammatory, antiviral and antifungal effects. Additionally, they can be useful in the synthesis of dyes and analytical reagents. There are more studies regarding the mechanism of action of isatin derivatives, which are the inhibition/modulation of proteases, translation initiation, neo-vascularisation and tubulin polymerization. All these studies drew attention to an isatin
and more investigations were performed to understand their structure activity relationship. Studies indicated that oxindoles could be obtained from synthetic or natural origin with fascinating biological activities. Until now, several oxindole derivatives were tested against different diseases such as cancer, inflammation and bacterial infection. Some of these derivatives exhibited high potential and reached clinical trials. Among the oxindoles have reached clinical trials, few of them already went into the market (Fig 1.4).

Figure 1.4 – Isatin based structures 1-3 which are already in the market.

Chronologically, Methisazone (1) has been used as anti-viral drug since 1965. After US-FDA approved the novel structure, Sunitinib (2) in 2006, which is kinase inhibitor for the treatment of gastrointestinal stromal tumors and advanced renal carcinoma. It is on the market as Sutent® by Pfizer. The mechanism of action is to inhibit multiple receptor tyrosine kinases, which actively work for tumor growth, pathogenic angiogenesis and metastatic progression of cancer. Recently, in 2009, a novel scaffold developed by Pfizer, Toceranib (Palladia) (3) was approved by US-FDA for skin-based cancer. Isatin scaffold has a great potential, however it still needs
extensive investigation in order to understand the SAR and biological activities, according to their different derivatives and varied pharmacological activities.

1.3 Spirooxindoles

1.3.1 Spirocyclic scaffold in nature

Spiro compounds are aromatic heterocyclic organic compounds with two ring systems. These rings are connected by a single (quaternary) carbon atom which is called as “spiro” carbon.

Spirocyclic compounds are found in the structures of natural products such as alkaloids, lactones or terpenoids isolated from plants and living organisms. The way of action and the therapeutic promise for molecules containing spirooxindoles have been a major impulsive force in order to develop novel advance synthesis paths. Moreover, due to the fact that spiro linked carbon possesses tetrahedral scaffold, connected rings stay almost perpendicular to each other. This characteristic structure provides unique conformational features.

In 1900, Adolf von Baeyer created the first spiran and proposed nomenclature of bicyclic hydrocarbons as “spirocyclane”. Since then, spirocyclic heterocycles have been studied further to become drug candidates because of their important structural features and promising biological activity on pharmacological and therapeutical studies.

In figure 1.5 spiro fused oxindole motifs from nature were indicated with the differences on their structures. As an example for spirocyclic scaffolds in nature, Coerulescine (4) was isolated from Phalaris coerulescens, blue canary grass and Horsfiline (5) was isolated from Horsfieldia superba Warb, Malaysian medical plant. These two examples show the simplest structure among the alkaloids. Nonetheless, they were excellent models to investigate novel compounds against human breast cancer cells. Spirotryprostatin A (6) and B (7), are indolic alkaloids isolated from the secondary metabolites of fungus Aspergillus fumigatus. These alkaloids have been
proved that they inhibit the G2/M progression of cell division in mammalian tsFT210 cells (isolated from a mouse mammary carcinoma cell line). Therefore, they have great potential as anti-cancer drugs. By using these alkaloids, several compounds and reactions were developed relatively to advance their activity\textsuperscript{35}.

![Figure 1.5 - Spiro-fused oxindole motifs from nature emphasized their key moieties with two different colors (blue and orange), pink points indicate spiro carbons.](image)

The oxindole alkaloid, Alstonisine, (8) is extracted from the plant *Alstonia acrophylla*\textsuperscript{36}. Similar to Spirotryprostatin compounds (6-7), spiro-pyrrolidine core scaffold of Alstonisine, (8) was used as pioneer structure to develop new possible MDM2 inhibitor (MI-888 in figure 1.7)\textsuperscript{37}. Different from those, (+)-welwitindolinone A (9) is an antifungal alkaloid that has a spiro-fused cyclobutyl oxindole. It was isolated from the marine blue-green alga *Hapalosiphon welwitschii* and used against multiple drug resistance. (+)-welwitindolinone A (9) functions as an anti-inflammatory agent while it is selective and potent p38α mitogen activated protein (MAP) kinase inhibitor. Lastly, Spirobrassinin (10) is a spiro-thiazolidinyl cruciferous phytoalexin which showed antiproliferative effect against T-jurkat leukemic cell lines\textsuperscript{38}(Fig 1.5).
Due to its complex and challenging mechanism, cancer is one of the most important concerns among the global diseases. Investigations for new anti-cancer compounds are continuously improving in order to get more selective and less toxic compounds. Like most pharmaceuticals, nature is an important guide for spirooxindole scaffold in order to design and develop novel compounds. Special characteristics and complex structures show significant connection to their biological activities. With the help of synthetic chemistry, these features are simulated and designed to advance cancer therapeutics.

1.3.2 Importance of Spirooxindoles

Spirooxindoles are particular structures derived from a member of natural products spirooxindole alkaloids, tryptamine. According to studies, the core structure gives promising biological activities with spiro center fused at the C3 position on oxindole base with diverted functional groups attached. The spirooxindole moiety is responsible for several biological activities as anti-HIV, antimicrobial, antimalarial, anti-cancer etc. (Fig 1.6).

![Figure 1.6 - Spirooxindole core structure and its wide range of biological activities.](image-url)
In the literature there are several examples of spirooxindoles with different biological activities. One of them is spiro-cyclohexyl Satavaptan (11), a vasopressin V2 receptor antagonist, designed by Sanofi-Aventis and reported as an orally active and highly selective for the treatment of hyponatremia. Another example, is compound MI-888 (12), which is capable of achieving rapid, long-lasting and complete tumor regression as p53/MDM2 inhibitor with a $K_i$ value of 0.44 nM in animal models of human cancer with oral administration. Additionally, Rhynchophylline (13) was described as an ion channel blocker which mainly acts on cardiovascular and central nervous system diseases. NITD609 (14) is a potent anti-malarial compound as an alternative to artemisinin derivatives. Recently, spiro-tetrahydro-β-carboline NITD609, has completed Phase II clinical trials in humans. Moreover, antipyretic compound SPX_F (15) is showing significant activity against neuroinflammation. Spirothiazolidinyl based compound was obtained as a potent 5-HT$_6$ antagonist (16) (Fig 1.7).

Figure 1.7 - Spirooxindole derivatives 11-16 with different biological activities.
Repetitive presence of spirocyclic oxindoles in numerous natural products and biologically active molecules allowed them to become known and interesting synthetic targets\textsuperscript{39}. In the past decade spirocyclic oxindoles have increasing demand in industry and attention in academia for development of their synthesis \textsuperscript{4}. Based on the recent statistics, the number of publications has increased unprecedentedly since 2010 because of notable attention on spirooxindole based structures (Fig 1.8).

Figure 1.8 - Number of publication for spirooxindoles between the years 2005 and 2015 (data taken from http://apps.webofknowledge.com/ in 2016).

Lately, a remarkable number of investigations has done to synthesize variety of spiro-based compounds with promising biological activities. Reduced molecular flexibility gives conformational restriction to the molecule leading to better results in terms of biological activities \textsuperscript{38,32}. Taking into consideration the results of biological activity studies, the most frequently reported spirooxindole moieties in the literature are active against cancer cell lines (Fig 1.9) \textsuperscript{39}. 

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To date, several important compounds have been discovered and some are already on the process of clinical trials. For instance, as mentioned before, MI-888 (12) is capable of achieving rapid, complete and durable tumor regression on experiments with different models of human cancer with oral administration (Fig 1.7) 46. One of the examples, CFI-400945 (17), inhibitor of Polo-like kinase 4 (PLK4) (Ki= 0.26 nM) was recently reported as a first-in-class, potent, selective and orally active anticancer agent. The first-in-human phase I trial demonstrated positive anti-tumor activity for multiple tumor types establishing targeted safety, tolerability, and pharmacokinetics. CFI-400945 (17) is considered a successful candidate for phase II trials 47. MI-219 (18) is an inhibitor of MDM2 presented by Feng et al. as a promising drug for the treatment of prostate cancer. The triple therapy with compound MI-219 (18), radiation and androgen deprivation therapy tumor growth, recurrence and death decreased significantly within the first 10 years of treatment 48. Additionally, with further improvements on pharmacokinetic profile of MI-219 (18) a new analog, SAR405838 (19), was developed. It demonstrated remarkable efficacy in humans as a single-agent in multiple
tumor models. Recent publication from Wang et al. reported that SAR405838 (19) possess Ki= 0.88 nM to human MDM2 protein (is >10, >50 and >1,000-times more potent than MI-219 (18), nutlin-3a and the p53 peptide, respectively) with good chemical stability and oral bioavailability. Studies against GTL-16 human gastric carcinoma models published by Li et al. revealed that compound SMU-B is orally bioavailable showing significant tumor growth inhibition and pharmacodynamics effect. Finally, one of the potential analogs, RO8994 (20), is a new generation of p53-MDM2 antagonists. RO8994 (20) was reported as a remarkable tumor growth inhibition in the wild-type p53 with IC50 of 5nM (HTRF binding assays) and 20nM (MTT proliferation assays). These structures are potential clinical development candidates for the treatment of various human cancers.

Focusing only in the anticancer properties of spirooxindoles, the diversity of heterocyclic motifs connected to C3 atom, 5 and 6 membered rings are the mostly studied types in 2011-12 (among 531 reports published) (Fig 1.10).
This unique structure, spiro [pyrrolidine-3,3’-oxindole] with 5 membered ring fused, is the focus of the new drug discovery and important synthetic targets because of its particular structure which possess a range of biological activities. Their selectivity and low toxicity features are also approved as an advantage for their anti-cancer potentials on cancer therapy.

1.4 1,3,4-oxadiazole scaffold

In pharmaceutical research area, heterocyclic ring systems have massive importance. One of the examples is oxadiazole (Fig 1.11), which includes in the chemical structure, two carbon atoms, two nitrogen atoms and one oxygen atom. Until now, oxadiazoles have already been used in different areas of medicine and pesticide chemistry. Additionally, they are used in material and polymer science. Recent reports took notice of 1,3,4-oxadiazoles cores as an important part of potential drug molecules. They have been frequently used as bioisosteric replacements for ester and amide functionalities being a part of drug like molecules.

![1,3,4-oxadiazole](image)

**Figure 1.11** - 1,3,4-oxadiazole.

1,3,4-oxadiazole was prepared, for the first time by Ainsworth, in 1965. Recently several reports have disclosed the spatial characteristics of the 1,3,4-oxadiazole scaffold and its importance in the medicinal chemistry. The importance of introducing 1,3,4-oxadiazole in drug discovery has several purposes. First of all, 1,3,4-oxadiazole can be used as an essential part of the pharmacophore because it contributes favorably to ligand binding. Second, by the virtue of its flat and aromatic structure, substituents can be placed in very specific and appropriate positions. Consequently, the combination of 1,3,4-oxadiazole moieties with potential drug candidates leads to changes in compound flexibility and polarity. These combinations change the effect on novel compounds’ metabolic profile and ability to engage in hydrogen bonding.
Generally, a variety of disease areas are interested in including 1,3,4-oxadiazole moiety in their novel drug candidates such as cancer, diabetes, obesity, inflammation and infection.  

There are some drugs in the market having 1,3,4-oxadiazole unit. Lately, AstraZeneca reported some examples of compounds containing an 1,3,4-oxadiazole moiety in the late stage clinical trials. In figure 1.12, two examples are included from different area of medicinal studies including oxadiazole scaffolds. Zibotentan (21) and Raltegravir (22) are already reported in the late stage clinical trials as anticancer agent and antiretroviral drug for the treatment of HIV infection, respectively.

1.5 **Scope of the thesis**

Previous work from our research group was focused on the development of novel spirooxindole scaffolds to modulate p53 activity. The first spirooxindole core developed was a structural analog of the spiro pyrrolidine oxindole scaffold present in the p53-MDM2 interaction inhibitor 23. In this scaffold the pyrrolidine ring was replaced by an isoxazoline ring. The synthesized compounds were shown to possess anticancer activity by disrupting the interaction between p53-MDM2, being compound 23 the most active in this series (Fig 1.13).
Further, optimization of spiroisoxazoline oxindoles led to the spiropyrazoline oxindole scaffold 24, which contains a nitrogen atom instead of an oxygen in the five membered ring (Fig 1.14) \(^{13}\). This change allowed to have one additional aromatic substituent (N-Ar) on the scaffold. Most compounds exhibited an increase of activity as anticancer agents comparing with the corresponding spiroisoxaline oxindoles. Moreover, compound 24 showed selectivity against MCF-7 over MDA-MB-231 breast cancer cell and the non-tumor HEK cell line.

Based on positive results of 23 and 24, another study was investigated for a novel library of spirooxadiazoline oxindoles \(^{13,57,56}\). In the new presented scaffold 25, five membered ring included [1,2,4]-oxadiazole core (Fig 1.15). This change revealed results as potentially more active than 23 and 24. The best results were obtained with halogen in 5\(^{th}\) or 6\(^{th}\) positions of the oxindoles and meta-halogens in both phenyl rings. Of the thirty-one compounds tested, compound 25 was the most active in HCT116.
"p53(+/+)" cell line with a 15.4-fold increase in potency over previous studies tested with compound 23\textsuperscript{58}.

![Chemical structure](image)

**Figure 1.15** - Example of previously studied spirooxadiazoline oxindoles.

Following these studies, in the present thesis, we aimed to develop a novel library of spirooxindole derivatives to be evaluated as potential anti-cancer agents. To date, no study has discussed the synthesis and the biological activities of 3',5'-diphenyl-spiro[indoline-3,2'-[1,3,4]oxadiazole]-2-one. Particularly, we intended to synthesize a novel library of compounds based on [1,3,4]-oxadiazole core and to diversify them by using different substituents on the phenyl rings (Fig 1.16). Compounds were further evaluated in cancer cell lines to ascertain their biological activities.
Figure 1.16 - Design of the spirooxadiazoline oxindoles.
2 Results and Discussion

2.1 Synthesis of a library of spirooxadiazoline oxindoles

Spirooxadiazoline oxindoles result from the combination of two potential anti-cancer scaffolds; oxadiazoline and oxindole. Oxadiazoline has been analyzed in various new synthesis methods for different pharmaceutical applications. Among the four possible isomers of oxadiazole, 1,3,4-oxadiazole is the most important in medicinal chemistry due to its various biological activities. Present in several drugs, it became an important scaffold for the development of novel drugs. Oxindole exists widely in tissues and fluids of mammals as well as in natural products extracted from a variety of plants, bacteria and invertebrates. They possess a diverse pharmacological profile due to the extensive range of biological activities. Spirooxadiazoline oxindoles containing a [1,2,4]oxadiazoline ring have already been studied in our research group. Based on the promising results obtained in our research group analysis with five membered ring spirooxindoles, in this thesis we decide to expand further these studies. For the first time a library of novel of spiro-oxindoles containing a [1,3,4]oxadiazoline ring was synthesized and evaluated as potential anti-cancer agents.

In this thesis, spiro[indoline-3,2’-[1,3,4]oxadiazoline]-2-ones synthesized with different substituents on phenyl groups in 3’ and 5’ positions and Cl or Br substituted on positions 5, 6 and 7 of the oxindole ring. Diversity was acquired by using different substituted aromatic aldehydes and arylhydrazines for the synthesis of the intermediaries. Additionally, commercially available derivatives of isatin were used for the final reaction. Retrosynthetic studies helped to construct the method of synthesis of spirooxadiazoline oxindoles (Scheme 1).
Spirooxadiazoline oxindoles 32 were synthesized by 1,3-dipolar cycloaddition between isatin derivatives 31 and nitrile imines 30 (formed in situ from hydrazonyl chlorides 29). Hydrazonyl chlorides 29 were synthesized using the procedure reported by Patel 59 and starting from hydrazones 28 (synthesized using the procedure reported by Hwu 60). The synthetic scheme is shown below (Scheme 2).

Scheme 1 - Retrosynthetic analysis of spirooxadiazolines.

Scheme 2 - Synthetic scheme to obtain spirooxadiazoline oxindoles 32. Reaction conditions: i) EtOH, rt; ii) NCS, DMS, DCM, -78°C; iii) TEA, DCM, rt.
2.1.1 Synthesis of hydrazones

Substituted hydrazones 28a-h were prepared by condensation reaction of phenylhydrazine derivatives 26 with benzaldehyde derivatives 27 in aqueous ethanol 20%. This core structure is a nitrogen-centered radical and it has been proved that can function as DNA-cleaving species. The $^1$H NMR spectra of intermediates 28 were in accordance with the ones reported in the literature for similar compounds.

Reaction mechanism is detailed in Scheme 4. The reaction involves the nucleophilic attack of the lone pair of electrons of one of the nitrogens of the hydrazine to the carbonyl carbon. Correspondingly, the desired hydrazone is obtained after the elimination of water.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>R²</th>
<th>R³</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>28a</td>
<td>H</td>
<td>H</td>
<td>75%</td>
</tr>
<tr>
<td>28b</td>
<td>m-Cl</td>
<td>m-Cl</td>
<td>95%</td>
</tr>
<tr>
<td>28c</td>
<td>p-MeO</td>
<td>H</td>
<td>72%</td>
</tr>
<tr>
<td>28d</td>
<td>p-Cl</td>
<td>H</td>
<td>96%</td>
</tr>
<tr>
<td>28e</td>
<td>H</td>
<td>m-Cl</td>
<td>89%</td>
</tr>
<tr>
<td>28f</td>
<td>H</td>
<td>o-Cl</td>
<td>92%</td>
</tr>
<tr>
<td>28g</td>
<td>m-Cl</td>
<td>o-Cl</td>
<td>90%</td>
</tr>
<tr>
<td>28h</td>
<td>H</td>
<td>p-Cl</td>
<td>92%</td>
</tr>
</tbody>
</table>

Reactions of hydrazones 28a-h were relatively fast (2-3h), performed in the dark at room temperature and gave excellent yields (72-96%) (Table 1). In the literature, yields of reference compounds indicated between 71-92%.

The synthesized compounds 28a-h were characterized by NMR. A typical $^1$H-NMR spectrum of compound 28a-h is exemplified by compound 28a. The N-H resonance signal is a broad singlet signal on lower field of spectrum (8.57 ppm). The resonance signal of N-H proton with no substituent hydrazone 28a is at 7.45 ppm. While for other hydrazones with substituents (28b-h) appeared as a broad singlet in a range between 7.70 - 9.70 ppm. The general distribution of proton chemical shifts indicates that aromatic group protons are displayed between 6.30 – 8.30 ppm. All synthesized hydrazone derivatives in the first step had two aromatic rings and, as expected, their signals were confirmed in the aromatic range of the NMR spectrum. In addition to the aromatic protons, another non-aromatic significant signal was one of the least shielded hydrogen from CH=N. This vinylic proton mostly overlapped with the resonance signals of phenyl ring B₂ and B₆ protons and gave multiplet signal around 7.71-7.55 ppm for compounds 28a, 28c and 28e. On the other hand, the rest of the hydrazones, proton from CH=N showed singlet signal. Additionally, a characteristic singlet at 3.82 ppm indicative of a -OMe group, was also on upper field on NMR spectrum at being the most shielded one.

a)
Figure 2.1 – a) $^1$H-NMR spectra for compound 28a with the enlargement of the spectra. b) $^1$H-NMR spectra for compound 28e with the enlargement of the spectra.
In the figure 2.1, are shown the difference between NH and CH=N chemical shifts for compounds 28a and 28e. For compound 28e, meta positioned halogen displayed inductive effect on the NH proton and signal appeared at 9.67 ppm (Fig 2.1). The proton signal for compound 28a was at 7.60 ppm. The proton of CH=N was observed between 7.70 – 7.62 ppm for 28a and at 7.92 ppm for 28e.

![Figure 2.2 - 13C-NMR spectrum for compound 28a](image)

In the 13C-NMR spectrum characteristic methine carbon connected to nitrogen gives a signal at 137.41 ppm for compound 28a. All the other hydrazone derivatives display small difference with carbon chemical shifts pending the substituents on their aromatic moiety. Carbons that can give different peaks were mainly the ones that have substituents. For example, when aromatic substituent -OMe was para positioned 28c, carbon that was connected to –OMe, displayed a strong paramagnetic effect at 160.10 ppm. However, without any substituent same carbon gave a signal at 128.55 ppm. In the figure 2.2, presented carbon peaks are corresponding with the reported results in the literature 62,63. For the other hydrazones the results were also consistent (see experimental procedure).
2.1.2 Synthesis of hydrazonyl chlorides

Hydrazonyl chlorides 29a-h were synthesized by reacting the appropriate N-
arylhydrazones with N-chlorosuccinimide-dimethyl sulphide complex, which was prepared in situ at -78°C. 

Hydrazonyl chlorides are widely used with oxindoles in 1,3-dipolar cyloaddition reactions which is one of the most chosen methods for 5 membered ring formation. In order to perform the chlorination, the respective hydrazone was prepared by using N-
chlorosuccinimide and dimethyl sulﬁde complex which is known as Corey-Kim reagent. As mentioned in the literature, N-chlorosuccinimide-dimethyl sulﬁde is a very reactive complex and is stable only at low temperatures. Nevertheless, reaction between hydrazone and Corey-Kim reagent occurs under extremely mild conditions and can be prepared in situ by an inexpensive method. Besides, this method was reported in good yields for hydrazonyl chlorides with short reaction time and simple procedure.

Initially, the halosulphonium salt was formed by N-chlorosuccinimide and dimethyl sulﬁde complex, which creates an electrophilic moiety to get a better leaving
Next, the nucleophilic attack leads up from the acidic N-H of hydrazone to give intermediate form of $N$-chlorosuccinimide. Sulfur with cation ion is more electrophilic and not willing to stay connected to the nitrogen, therefore electron from the neighbor double bond helps dimethylsulfide group to leave and forms the stable cation on carbon. The stable cation undergoes attack by the Cl- to form alkyl halide compound. The reason why benzylic cation is more stable is explained with the presence of adjacent nitrogen atom. This cation can give phenylazobenzyl chloride which after tautomerise to $N$-phenylhydrazonyl chlorides (Scheme 6) 29.

Table 2 - Scope of hydrazonyl chlorides and their yields.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$R^2$</th>
<th>$R^3$</th>
<th>Yield</th>
</tr>
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<tbody>
<tr>
<td>29a</td>
<td>H</td>
<td>H</td>
<td>69%</td>
</tr>
<tr>
<td>29b</td>
<td>$m$-Cl</td>
<td>$m$-Cl</td>
<td>61%</td>
</tr>
<tr>
<td>29c</td>
<td>$p$-MeO</td>
<td>H</td>
<td>58%</td>
</tr>
<tr>
<td>29d</td>
<td>$p$-Cl</td>
<td>H</td>
<td>83%</td>
</tr>
<tr>
<td>29e</td>
<td>H</td>
<td>$m$-Cl</td>
<td>48%</td>
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<tr>
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<td>H</td>
<td>$o$-Cl</td>
<td>92%</td>
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<td>29g</td>
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<td>$o$-Cl</td>
<td>90%</td>
</tr>
<tr>
<td>29h</td>
<td>H</td>
<td>$p$-Cl</td>
<td>92%</td>
</tr>
</tbody>
</table>
The products were obtained in moderate to excellent yields (48-92%). The structure of the synthesized compounds 29a-h was confirmed by NMR and compared with literature \(^{59,61}\).

The \(^1\)H-NMR spectrum of hydrazonyl chlorides are relatively similar to the ones obtained for the corresponding hydrazones but with one less proton signal because of the Cl addition. Nevertheless, the presence of a chlorine atom shielded signals of H-B2 and H-B6 comparing the same positioned protons NMR spectra of hydrazonyl chloride (29a) with the starting material hydrazone (28a).

**Figure 2.3 - \(^1\)H-NMR spectra of hydrazone (28a) overlapped with spectra of hydrazonyl chloride(29a).**

In order to understand the differences, both hydrazone 28a and hydrazonyl chloride 29a NMR spectra examples were overlapped as seen in the above image (Fig 2.3). The \(^1\)H NMR spectrum confirmed the difference when Cl atom is connected to the carbon of CH=N 29a or not 28a. Overall, vicinal protons in both compounds are the only and the most affected ones. For example, the single proton of N-H appeared as a broad singled but it was shifted downfield and gave signals between 8.00-9.00 ppm. Moreover, the ortho positioned aromatic hydrogens (H-B2 and H-B6) in compound 29a
appeared more shielded than the proton of N-H and gave doublet signal instead of multiplet. This was also because of the absent proton connected to the imine carbon. For instance, -OMe protons from the compound 29c did not show big differences when it was compared with protons from the compound 28c. A characteristic singlet of -OMe group for compound 29c determined at 3.90 ppm, so signal moved slightly to lower field on NMR spectrum, but it was still the most shielded one.

The $^{13}$C NMR spectra confirmed the presence of two aromatics and an imine group. They revealed the presence of 13 carbon signals, comprising three quaternary carbons (two aromatic sp3 carbons at 172.0 ppm and 174.2 ppm, ten carbons appear at 71.4 ppm, one imine at 42.6 ppm). Comparing with $^1$H-NMR spectra, observed signals in $^{13}$C-NMR spectra for hydrazone and hydrazonyl chloride also did not give surprising changes. The visible difference in chemical shift was mainly for the quaternary carbon CCl=N 61. For the other hydrazonyl chlorides the results were also consistent (see experimental procedure).

2.1.3 Synthesis of 3',5'-diphenyl-spiro[indoline-3,2'-[1,3,4]oxadiazoline]-2-one

![Scheme 7 - Synthesis of spirooxadiazoline oxindoles 32a-I.](image)

Cycloaddition reaction of nitrile imine to C=O groups was indicated as a successful method for oxadiazole formation. Different methods have been reported for the synthesis of spirooxindole scaffold until now, however there is still limited research for spirooxindoles containing an oxadiazoline moiety 67. One of the examples is the reaction of isatin with carbohydrazine 68 and the other is with cyanoacetic acid hydrazine 69. However, both include multistep reaction methods and show several
disadvantages such as, moderate yield of products, extended reaction time, difficult reaction conditions with high temperature. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
<th>Yield</th>
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<td>H</td>
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</tr>
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<td>H</td>
<td>p-MeO</td>
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</tr>
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<td>m-Cl</td>
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</tr>
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<td>m-Cl</td>
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</tr>
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<td>H</td>
<td>p-Cl</td>
<td>69%</td>
</tr>
<tr>
<td>32l</td>
<td>5-Br</td>
<td>H</td>
<td>p-Cl</td>
<td>66%</td>
</tr>
<tr>
<td>32m</td>
<td>6-Cl</td>
<td>p-Cl</td>
<td>H</td>
<td>35%</td>
</tr>
<tr>
<td>32n</td>
<td>H</td>
<td>H</td>
<td>m-Cl</td>
<td>49%</td>
</tr>
</tbody>
</table>

In this study, we extended the scope of this reaction with different isatins and hydrazonyl chlorides. As a result, the corresponding spirooxindoles were synthesized from moderate to high yields. Therefore, spirooxadiazoline oxindoles were synthesized by using a simple and efficient method; 1,3-dipolar cycloaddition reaction between indoline-2,3-diones and hydrazonyl chlorides in the presence of Et₃N in CH₃Cl₂ at room temperature. The comparison with the other methods indicates advantages of this method namely, using available starting materials, mild reaction condition, relatively short reaction time and high yields of product.
Reactions in the thesis have been performed by isatin derivatives and after spirooxindole structure formation, oxindole motif existed in the final scaffold. Isatins include multiple bond system, namely “dipolarophile” and hydrazonyl chloride derivatives are zwitterionic in all-octet resonance structures 30 which are seen ambivalent on 1- and 3-positions, namely “dipole”. These structures show both electrophilic and nucleophilic activity. 1,3-dipolar cycloaddition reaction, which is also known as [3+2] cycloaddition leads to 5-membered heterocycles with remarkably wide range of utility in the synthesis (Scheme 9).

Scheme 8 - Mechanism of spirooxadiazoline reaction.

Scheme 9 – Reaction mechanism of 1,3-dipolar cycloaddition reaction.
The reaction starts in the presence of the base, triethylamine, added to a mixture of hydrazonyl chloride and isatin in dichloromethane. Base abstracts the proton of nitrogen from the corresponding hydrazonyl chloride and undergoes to the formation of nitrile amine, which is generated in situ. After 1,3-dipolar cycloaddition reaction takes place and formed the 5 membered oxadiazoline ring (Scheme 10). The reaction took place at room temperature for 16h (overnight) to afford 3',5'-diphenyl-spiro[indoline-3,2'-[1,3,4]oxadiazoline]-2-one in 78% yield.

This year, Alizadeh et al. published the same spirooxindole scaffolds as combination of spirooxindole and [1,3,4]-oxadiazole structures. The paper was focused on the synthesis of spiro[1,3,4]-oxadiazoline oxindoles, but biological studies were not performed.

**Figure 2.4** - $^1$H-NMR spectrum of compound 32a in acetone- $d_6$ and numbered structure. (Full spectra is presented in Appendix)

The $^1$H-NMR spectra of compound 32a was performed and is presented in the figure 2.4. The NH proton signal showed up on the lower field of the spectrum. Comparing with the respective hydrazonyl chloride, the final compound 32a showed an increase of aromatic porton signals, which suggests that 1-3 dipolar cycloaddition reaction occurred. Analyzing the $^1$H-NMR spectrum, the presence of the cloro atom in the 5th position of the oxindole ring gave vicinal hydrogen C$_4$ on the less shielded field, determined at 7.62 ppm. Other protons are C$_6$ and C$_7$ which were more shielded and determined at 7.16 ppm and at 6.85 ppm respectively. Moreover, as mentioned before NH proton showed up on the lower field at 10.10 ppm. Other signals for protons, which
were on the aromatic rings, did not appear so different from the signals of corresponding hydrazonyl chloride.

Figure 2.5 - $^1$H-13C HMQC spectrum of compound 32a in acetone-$d_6$.

Figure 2.6 – $^{13}$C-NMR spectrum of compound 32a in acetone-$d_6$. 

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The $^{13}$C NMR and HMQC spectra confirmed the presence of the spiro, carbonyl, nitrile and two aromatic groups. In order to confirm the position of carbons HMQC spectrum assisted to clarify aromatic and especially the isatin core carbons (Figure 2.5). For compound 32a, they revealed the existence of 21 carbon signals, comprising eight quaternary carbons; spiro carbon at 95.7 ppm, two aromatic carbons at 129.0 ppm and 143.1 ppm, one carbon on oxadiazoline core at 152.7 ppm, carbonyl carbon at 171.6 ppm, two carbons on indoline core at 126.6 ppm and 142.4 ppm and carbon on aromatic ring of indoline 125.7 ppm. Additionally, thirteen methines on aromatic rings appeared between 113.8 – 133.4 ppm (Figure 2.6).

Scheme 10 - Regioselectivity of 1,3-dipolar cycloaddition reactions with spirooxadiazoline oxindoles.

Stereochemistry is necessary for pharmacology and has a huge impact on ADME of therapeutic molecules 39. Taking into consideration the stereoselectivity of 1,3-dipolar cycloaddition reactions, there are two possible regioisomeric oxadiazoline formations based on the electronic and steric effects of reactants. Nevertheless, based on following collected data, we confirmed that the regioisomer obtained was the spiro[indoline-3,3’-[1,3,4]oxadiazoline]-2-one. First, when the vicinal atoms are oxygen and nitrogen, the $^{13}$C NMR spectrum indicated that spiro carbon signal was observed around 96.04 ppm. Furthermore, searching through the literature, in the oxadiazoline ring the chemical shift of the carbon is around 150.0 ppm but for similar spiro compounds resonance signals move more shielded field ranging between 94.6-96.0 ppm.
For the alternative regioisomer with vicinal atoms are oxygen and carbon, the $^{13}$C NMR spectrum indicates that spiro carbon signal shows up around 87.6-89.8 ppm $^{70}$. Additionally, chemical shift of carbon atom from oxadiazoline C=N appeared around 152.0-155.0 ppm whilst in literature 155.4-157.0 $^{71}$ (Scheme 11). This difference is enough to understand if obtained compound is the desired one or not.
2.2 Biological Evaluation

2.2.1 Cellular activity and structure-activity relationships (SAR)

The anti-proliferative activity of compounds 32a-r was tested in vitro against different cancer cell lines: an isogenic matched pair of wild type p53 and deleted human colorectal cancer cell lines [HCT116 p53(+/+) and p53(-/-)], and human colorectal adenocarcinoma cell lines [SW620 (mut p53)].

Table 4 - Tested compounds and their substituents

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R^1</th>
<th>R^2</th>
<th>R^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>32a</td>
<td>5-Cl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>32b</td>
<td>7-Br</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>32c</td>
<td>6-Cl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>32d</td>
<td>7-Cl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>32e</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>32f</td>
<td>5-Br</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>32g</td>
<td>6-Cl</td>
<td>H</td>
<td>p-MeO</td>
</tr>
<tr>
<td>32h</td>
<td>6-Cl</td>
<td>m-Cl</td>
<td>m-Cl</td>
</tr>
<tr>
<td>32i</td>
<td>5-Br</td>
<td>m-Cl</td>
<td>m-Cl</td>
</tr>
<tr>
<td>32j</td>
<td>5-Cl</td>
<td>m-Cl</td>
<td>m-Cl</td>
</tr>
<tr>
<td>32k</td>
<td>5-Cl</td>
<td>H</td>
<td>p-Cl</td>
</tr>
<tr>
<td>32l</td>
<td>5-Br</td>
<td>H</td>
<td>p-Cl</td>
</tr>
</tbody>
</table>

Twelve compounds were synthesized with different substituents attached to the spirooxadiazoline oxindole core structure and tested for their biological anti-tumor activity. Results are reported in detail below for compounds 32a-l.

Introduction of electron-withdrawing halogens on the benzene ring of indolin-2-one was reported to be more favorable for increasing the activity on HCT116 p53(+/+) and HCT116 p53(-/-). However, they were non-cytotoxic at concentrations up to 50 µM. The activity varied with the difference in position of substituents of the indolin-2-one. The best results were obtained with 5 and 6 positioned halogens on the oxindole ring.
Based on the first screening, eight compounds did not show any significant activity for the cell line with p53 (HCT116 p53+/+). Only compound 32g showed more potency and decreased the cell viability more than the other compounds but viability was still above 50% even with 50 µM concentration. Similar results were obtained for compounds tested in HCT116 p53−/− cancer cell lines. As seen in the figure 2.7, almost all compounds had slightly decreased in their viability with increasing concentration.
However, none of them decreased viability below 50% with 5-fold and 10-fold increase in concentration.

All compounds tested against HCT116 p53\(^{+/+}\) and HCT116 p53\(^{-/-}\) cancer cell lines were not active up to 50 µM and a lack of selectivity was observed between these cancer cell lines. On the other hand, interestingly there was no growth or specific increase in the amount of cancer cells although they did not show any significant decrease.

![SW620 cell line](image)

**Figure 2.8** - Cell viability (%) determined by the MTS method after 72h of exposure. Results are given in % relative to control and represent means with 95% confidence intervals of three independent experiments performed in triplicate and carried out independently.

Compounds 32a-h were more active for SW620 cell lines than in both HCT cell lines. Moreover, three of these derivatives 32a, 32b, 32e, 32f had higher activity than the others (Fig 2.8). As common feature, all these compounds, do not have any substituents on respective aromatic rings but oxindole ring was substituted with Br or Cl in different positions. Comparing anti-tumor activities for compounds 32a, 32b, 32e, 32f, three independent experiments were performed in triplicate and carried out independently. IC\(_{50}\) values were calculated based on these experiments.
Figure 2.9 – Cell viability (%) for compounds 32b and 32e in SW620 cell line for 72h of exposure. Results are given in % relative to control and represent means with 95% confidence intervals of three independent experiments performed in triplicate and carried out independently.

As seen in the figure 2.9 viability decreased as desired and IC$_{50}$ values of these compounds were calculated. Compounds 32b and 32e possessed Br and Cl in positions 7 and 6 of the oxindole respectively and no substituents on the other aromatic rings. For compound 32b and 32e IC$_{50}$ values were calculated as 26.47 µM and 7.98 µM. However, it was proved that it has potential on mutated cancer cell lines and this will be further investigated in future studies.

Figure 2.10 - Cell viability (%) for compounds 32a and 32f in SW620 cell line for 72h of exposure. Results are given in % relative to control and represent means with 95% confidence intervals of three independent experiments performed in triplicate and carried out independently.

Compounds 32a and 32f have the highest activity against SW620 cancer cell line (Fig 2.10). Remarkably, compounds showed the best potent having the IC$_{50}$ values of 6.42 µM for 32a and 4.59 µM for 32f. Both scaffolds have halogen in position 5th of the oxindole and unsubstituted aromatic rings. Additionally, one of the best potential
compounds (32a) was further investigated in CCD-18Co human normal colon fibroblasts in order to determine its toxicity (Fig 2.11).

**Figure 2.11** - Cell viability (%) for compound 32a in SW620 and CCD-18Co cell lines for 12.5 µM and 72h of exposure. Results are given in % relative to control and represent means with 95% confidence intervals of three independent experiments performed in triplicate and carried out independently.

Noteworthy, results confirmed that with four different concentrations, 32a did not result in any toxicity against normal colon cell CCD-18Co up to 50 µM (Fig 2.11). This result was positive for future studies.
After having the best results for compounds 32a and 32f, which were Cl and Br substituted on the 5th position of oxindole core, more derivatives were synthesized and screened to test their biological activities. Therefore, compounds with Cl or Br substituted on the 5th position of oxindole core remained same for all derivatives but substituents on aromatic ring were changed. Compounds 32i, 32j, 32k and 32l were recently tested in SW620 and HCT116 p53+/+ cell lines with only two different concentrations. The aim of this test was to screen the potential of new compounds in these different cell lines. HCT116 p53+/+ was chosen in order to understand if the derivatives of compounds are also only active against mutated cell lines. Regarding the HCT116 p53+/+ cell viability, results were as expected and none of the tested compounds revealed activity up to 20 µM. On the other hand, the compounds displayed remarkably low cell viability on mutated cell line, SW620, not only for 20 µM but also for 10 µM concentrations used. Since there were not enough replicates of this experiment, IC50 value was not possible to calculate. However, these high potential anti-cancer compounds will be further evaluated.
SAR analysis was concluded as seen below in order to have an idea about the key parts of the compounds for their activity (Fig 2.13).

**Figure 2.13** – SAR studies for the result and discussion.
CHAPTER III - CONCLUSION AND FUTURE WORK
3 Conclusion

In conclusion, in this thesis new libraries of spiro[indoline-3,2′-[1,3,4]oxadiazol]-2-ones were synthesized from the reaction of isatin derivatives and hydrazonyl chlorides by using the 1,3-dipolar cycloaddition reaction.

On the way of discoveries for a new generation of effective and potential anti-cancer compounds, spirooxadiazoline oxindoles have come out with their spatial scaffolds and potential biological activity. In this thesis for the first time oxindole moiety was merged with [1,3,4]-oxadiazoline moiety. Reactions were successful and completed with high yields. Moreover, compounds were tested *in vitro* against different cancer cell lines: an isogenic matched pair of wild type p53 and deleted human colorectal cancer cell lines [HCT116 p53(+/+) and HCT116 p53(-/-)], and human colorectal adenocarcinoma cell lines [SW620 (mut p53)]. Results obtained demonstrated that compounds 32a-h display activity against SW620 (mut p53) cell lines. Specifically, compounds 32a, 32e and 32f showed remarkable results with IC_{50} 6.42 µM, 7.98 µM and 4.59 µM respectively. Besides, in order to determine if compounds are toxic against normal cell lines, compound 32a was tested in CCD-18Co human normal colon fibroblasts. Results revealed no toxicity. Compound 32a was confirmed by having a high activity against SW620 cancer cell lines whilst having no toxic impact on normal fibroblast cells. Additionally, according to screening tests against HCT p53^{+/+} and SW620 cell lines, compounds 32i-l showed high anti-cancer potential against SW620 cell lines more than HCT p53^{+/+}. However, these results must be further investigated. As a result, compounds showed more activity in mutated SW620 cell line than others. The mechanism of action is perhaps working in the mutated form, so extented library of spirooxadiazolines can be further tested to improve the antiproliferative activity in mutated SW620 cell line. Besides, compounds with the best activity against cancer cell lines were with Cl or Br substituted on their oxindole core.

According to the promising results that are reported in this thesis, in terms of future directions, a new library can be synthesized with the indoline-2,3-diones Cl or Br in position 5. In literature to increase the anti-cancer activity, the importance of halogen
in 5\textsuperscript{th} position has been reported\textsuperscript{72, 73}. Based on good results from \textit{in} vitro studies, increase in potency was obtained without any substituents on aromatic rings. However, combining 5-Cl or 5-Br isatins with different hydrazonyl chlorides with variety of halogens or more bulky groups on phenyl groups should be evaluated to get more interesting results. Besides, best compounds can be tested in different cancer cell lines in order to see the difference in activity. Additionally, we performed the first screening in mutated cell line SW620 for compounds \textbf{32i}, \textbf{32j}, \textbf{32k} and \textbf{32l} which showed a promising decrease in viability. Still further experiments have to be performed to confirm our hipotesis.
CHAPTER IV - EXPERIMENTAL WORK
4 General Methods

4.1 Reagents and Equipment

Reagents and solvents were obtained from commercial suppliers and used with no further purification. The solvents were of analytical grade and in most cases dried by distillation when the reaction conditions required a dry solvent. DCM was dried by distillation from calcium chloride. The water used in reactions and preparation of preparative was purified in the laboratory. All deuterated solvents were purchased from Sigma-Aldrich.

For monitoring the reactions, analytical thin layer chromatography Analysis Merck Silica Gel 60 F254 aluminum plates were used. The plates were visualized using UV light. For purification, flash column chromatography with Merck Silica Gel (200-400 mesh) silica were used. In order to obtain a finer purification, glass plates coated with silica Merck Silica Gel 60 GF254 were used to perform preparative thin layer chromatography.

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker 300 Ultra-Shield. $^1$H nuclear magnetic resonance spectra were recorded at 300 MHz and $^{13}$C nuclear magnetic resonance spectra were recorded at 75 MHz. $^1$H and $^{13}$C NMR chemical shifts are reported in parts per million (ppm, $\delta$) referenced to the solvent used and the proton coupling constants $J$ in hertz (Hz). Multiplicities are given as: s (singlet), bs (broad singlet), d (doublet), dd(doublet doublet), t (triplet), dt (doublet triplet), q (quartet), m (multiplet). Spectra were assigned using appropriate COSY, DEPT, HMQC and HMBC sequences. Elemental analysis was performed in a Flash2000 ThermoScientific elemental analyzer and are within ±0.4% of theoretical values. Mass spectra were analyzed using MassLynxV 4.1 software. For determination of melting points Kofler camera Bock monoscope M. was used.

Besides, the synthesis of compounds 28d-h and 29d-h used for the synthesis of the target compounds described in this master thesis were used from studies of Carlos Ribeiro.
4.2 Synthetic Procedure and Product Characterization

4.2.1 General procedure for the synthesis of hydrazones (28a-h)

A phenylhydrazine derivative (1.0 equiv) was mixed with benzaldehyde derivative (1.2 equiv) in aqueous ethanol 20% (2mL/mmol of phenylhydrazine) and stirred at room temperature in the dark for 2-3 hours. The precipitate formed was filtered and washed with aqueous ethanol 20%. For the synthesis of compounds 28b, 28e and 28h, the phenylhydrazine used was in hydrochloride form, so it was necessary to add triethylamine (1.0 equiv) 15 minutes before adding the corresponding benzaldehyde (adapted from 60). For characterization, chloroform-d was used as a solvent.

4.2.1.1 Synthesis of 1-benzylidene-2-phenylhydrazine (28a)

Synthesized according to the general procedure; 2.35 g benzaldehyde (18.49 mmol, 1eq.) was mixed with ethanol (20%) (40 ml). Then 2.0 mL phenylhydrazine (22.19 mmol, 1.2eq.) was added. Synthesized according to the general procedure, compound 28a was obtained as a brown solid (2.7 g, 76 % yield).

**MP:** 156-158 ºC (mp lit. 154 ºC 62, 158-160 ºC 74),

**1H NMR** (400 MHz, CDCl3) δ (ppm): 7.70 – 7.62 (m, 3H, CH=N, H-B2,6), 7.60 (br s, 1H, NH), 7.45 – 7.35 (m, 2H, H-B3,5), 7.35 – 7.24 (m, 3H, H-B4, H-C3,5), 7.13 (dd, J = 8.5, 1.0 Hz, 2H, H-C2,6), 6.96 – 6.85 (m, 1H, H-C4). NMR spectra are in accordance with the ones described in the literature for this compound 62, 63.
4.2.1.2 Synthesis of 1-(3-chlorobenzylidene)-2-(3-chlorophenyl)hydrazine (28b)

Synthesized according to the general procedure; 1.041 g 3-Cl phenylhydrazine (5.585 mmol, 1eq.) was mixed with ethanol (100%) (this time without water) (2.4 ml) and 0.76 mL TEA were added to mixture. After 0.76 mL 3-Cl benzaldehyde (6.702 mmol, 1.2eq.) was added to the mixture. Synthesized according to the general procedure, compound 28b was obtained as a light pink solid (1.4 g, 95 % yield).

Mp: 98-100 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) (ppm): 7.70 (br s, 1H, NH), 7.66 (s, 1H, H-B\(_2\)), 7.62 (s, 1H, CH=N), 7.50 (dt, \(J = 6.9, 1.8\) Hz, 1H, H-B\(_6\)), 7.35 – 7.27 (m, 2H, H-B\(_{4,5}\)), 7.22 – 7.15 (m, 2H, H-C\(_{2,5}\)), 6.92 (ddd, \(J = 8.2, 2.1, 0.9\) Hz, 1H, H-C\(_6\)), 6.85 (ddd, \(J = 8.1, 2.1, 1.0\) Hz, 1H, H-C\(_4\)). NMR spectra are in accordance with the ones described in the literature for this compound\(^64\).

4.2.1.3 Synthesis of 1-(4-methoxybenzylidene)-2-phenylhydrazine (28c)

Synthesized according to the general procedure; 1.00 g phenylhydrazine (9.25mmol, 1eq.) was added to 1.35 mL 4-OMe benzaldehyde (11.1 mmol, 1.2eq.). After ethanol (20%) (20.11 ml) was added to mixture. Synthesized according to the general procedure, compound 28c was obtained as a light pink solid (3592.3 mg, 72 % yield).

Mp: 116-118 °C (mp lit. 115 °C\(^75\), 120 °C\(^74\)); \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) (ppm): 7.63 – 7.54 (m, 3H, CH=N, H-B\(_{2,6}\)), 7.46 (br s, 1H, NH), 7.31 – 7.21 (m, 2H H-C\(_{3,5}\)), 7.12 – 7.05 (m, 2H, H-C\(_{2,6}\)), 6.93 – 6.88 (m, 2 H-B\(_{3,5}\)), 6.88 – 6.81 (m, 1H, H-C\(_4\)), 3.82 (s, 3H, OCH\(_3\)). NMR spectra are in accordance with the ones described in the literature for this compound\(^62, 64\).
4.2.2 General procedure for the synthesis of hydrazonyl chlorides (29a-h)

To a solution of N-chlorosuccinimide (NCS) (3.0 equiv) in dichloromethane (DCM) (3.5 mL/mmol of hydrazone) at 0 °C was added dimethyl sulfide (DMS) (6.0 equiv) over 5 minutes. After stirring for 15 minutes, the reaction was further cooled to –78 °C. Then the corresponding hydrazone 28a-h (1.0 equiv) dissolved in CH$_2$Cl$_2$ (1 mL/mmol of hydrazone) was added. The reaction was stirred at –78°C for 1h, and then slowly allowed to warm to room temperature over 3 h. The reaction was quenched by addition of cold water. The organic layer was then washed with brine (1x), saturated sodium sulfite aqueous solution (2x), and water. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated to afford the corresponding hydrazonoyl halide (adapted from 59,61). For characterization, chloroform-d was used as a solvent.

4.2.2.1 Synthesis of N'-phenylbenzohydrazonoyl chloride (29a)

Mixed of NCS (4.086 g) with DCM (41.36 mL) at 0°C in ice bath. Then added DMS (5.63mL) over 5 minutes. After stirring for 15 minutes, the reaction was further cooled to -78°C and hydrazone (2.0084g) was added. Synthesized according to the general procedure, compound 29a was obtained as a brown solid (1612.2 mg, 69 % yield).

Mp: 128-130 °C (mp. lit 129-130 °C 76 129-131 °C 77); $^1$H NMR (400 MHz, CDCl$_3$) δ (ppm): 8.05 (br s, 1H, NH), 7.96 – 7.89 (m, 2H, H-B$_2$), 7.46 – 7.35 (m, 3H, H-B$_{3,4,5}$), 7.35 – 7.28 (m, 2H, H-C$_3$), 7.22 – 7.15 (m, 2H, H-C$_{2,6}$), 6.95 (t, J = 7.3 Hz, 1H, H-C$_4$).

NMR spectra are in accordance with the ones described in the literature for this compound 61.
4.2.2.2 Synthesis of 3-chloro-N’-(3-chlorophenyl)benzohydrazonoyl chloride (29b).

Mixed of NCS (1.81 g) with DCM (18.3 mL) at 0°C in ice bath. Then added DMS (2.0 mL) over 5 minutes. After stirring for 15 minutes, the reaction was further cooled to -78°C and hydrazone (1.2 g) was added. Synthesized according to the general procedure, compound 29b was obtained as a dark brown-reddish solid (1648.7 mg, 61 % yield). 

**Mp:** 52-54 °C; $^1$H NMR (300 MHz, CDCl$_3$) δ (ppm): 8.08 (br s,1H, NH), 7.91 – 7.87 (m, 1H, H-B$_2$), 7.83 – 7.77 (m, 1H, H-B$_6$), 7.38 – 7.33 (m, 2H, H-B$_{3,5}$), 7.24 – 7.17 (m, 2H, H-C$_{2,6}$), 7.04 – 6.98 (m, 1H, H-C$_6$), 6.93 (ddd, J = 8.0, 1.9, 0.9 Hz, 1H, H-C$_4$). NMR spectra are in accordance with the ones described in the literature for this compound.$^{61}$

4.2.2.3 Synthesis of 4-methoxy-N’-phenylbenzohydrazonoyl chloride (29c).

Mixed of NCS (3.56 g) with DCM (35.73 mL) at 0°C in ice bath. Then added DMS (3.9 mL) over 5 minutes. After stirring for 15 minutes, the reaction was further cooled to -78°C and hydrazone (2.00 g) was added. Synthesized according to the general procedure, compound 29c was obtained as a dark brown semi-solid (1431.2 mg, 58 % yield).

**MP:** 134-135 °C (mp. lit 133-134 °C $^{59}$)

$^1$H NMR (300 MHz, CDCl$_3$) δ (ppm): 9.89 (br s, 1H, NH), 7.87 – 7.82 (m, 2H, H-B$_2$), 7.36 – 7.30 (m, 2H, H-C$_{3,5}$), 7.21 – 7.18 (m, 2H, H-C$_{2,6}$), 7.04 – 6.98 (m, 2H, H-B$_{3,5}$), 6.82 – 6.75 (m, 1H, H-C$_4$), 3.90 (s, 3H, OCH$_3$). NMR spectra are in accordance with the ones described in the literature for this compound.$^{61}$
4.2.3 General procedure of spirooxadiazoline oxindole reactions

A solution of a corresponding hydrazonyl chloride (2.0 equiv) in DCM (1 ml/mmol of isatin) was added to corresponding isatin (1.0 equiv) in the room temperature and under argon atmosphere. The reaction was stirred for 15 mins before addition of TEA (2.0 equiv). TEA was added in a dropwise manner. After addition completed, reaction stirred overnight under argon atmosphere until total consumption of the starting isatin. The organic (DCM) phase was washed twice with saturated aqueous solution NaCl, dried with anhydrous sodium sulfate, filtered and evaporated. After purification by flash chromatography using ethyl AcOEt/n-hexane as eluent. Compound was washed with ether in order to get the final product.

4.2.3.1 Synthesis of 5-chloro-3',5'-diphenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (32a)

Following the general procedure, 253.9 mg of 29a (1.101 mmol, 2 eq) was added to 100.4 mg of 5-Cl isatin (0.551 mmol, 1eq.) which was dissolved in DCM (3 mL). Then, 153 mL of TEA (1.101 mmol, 2 eq.) was added added in a dropwise manner. Synthesized according to the general procedure, compound 32a was obtained as a light yellow solid in a 78% yield. Reaction time: 64h.
MP 200 – 202 °C; IR ν_max (KBr, selected peaks): 3251 (NH), 1734 (C=O), 1599 (C=N), 1495, 1474, 1375, 1205, 1144, 767, 688 cm⁻¹. ¹H NMR (300 MHz, Acetone-d₆) δ 10.09 (br-s, 1H, NH), 7.91 – 7.86 (m, 2H, H-A₂,₆), 7.63 (d, J = 2.2 Hz, 1H, H-4), 7.56 – 7.50 (m, 4H, H-6, H-A₃,₄,₅), 7.22 – 7.15 (m, 3H, H-B₃,₅, H-7), 6.93 – 6.88 (m, 2H, H-B₂,₆), 6.88 – 6.81 (m, 1H, H-B₄) ppm.; ¹³C NMR δ (75 MHz, Acetone-d₆) δ 171.61 (C=O), 152.76 (C=N), 143.19 (ArCq-B), 142.40 (ArCq-7a), 133.47 (ArC), 131.73 (ArC), 130.05 (ArC), 129.71 (ArC), 129.08 (ArCq-A), 127.15 (ArC), 127.05 (ArC), 126.60 (Cq-3a), 125.73 (ArC), 121.86 (ArC), 114.86 (ArC), 113.89 (ArC), 95.72 (Cspiro) ppm. ESI-MS m/z: 376.2 [M+H]^+. 

4.2.4 Synthesis of 7-bromo-3',5'-diphenyl-3'H-spiro[indoline-3,2']-[1,3,4]oxadiazol]-2-one (32b)

Following the general procedure, 153.10 mg of 29a (0.66 mmol, 2 eq) was added to 74.9 mg of 7-Br isatine (0.332 mmol, 1eq.) which was dissolved in DCM (3 mL). Closed round bottom flask and put the Nitrogen gas balloon. Then, 0.09 mL of TEA (0.66 mmol, 2 eq.) was added. Synthesized according to the general procedure, compound 32b was obtained as a dark yellow solid in a 46% yield. Reaction time: 16h. MP 235 - 237°C; IR ν_max (KBr, selected peaks): 3421 (NH), 1747 (C=O), 1598 (C=N), 1495, 1472, 1373, 1288, 1144, 784, 750, 687 cm⁻¹. ¹H NMR (300 MHz, Acetone-d₆) δ 10.23 (br-s, 1H, NH), 7.94 – 7.83 (m, 2H, H-A₂,₆), 7.69 (d, J = 8.2 Hz, 1H, H-4), 7.58 – 7.46 (m, 4H, H-6, H-A₃,₄,₅), 7.24 – 7.15 (m, 2H, H-B₃,₅), 7.15 – 7.07 (m, 1H, H-5), 6.93 – 6.81 (m, 3H H-B₂,₄,₆) ppm.; ¹³C NMR δ (75 MHz, Acetone-d₆) δ 171.67 (C=O), 152.80 (C=N), 143.18 (ArCq-B), 142.91 (ArCq-7a), 136.34 (ArC), 131.77 (ArC), 130.05 (ArC), 129.71 (ArC), 130.07 (ArCq-A), 129.74 (ArC), 127.02 (ArC), 126.61 (Cq-3a), 126.06 (ArC), 125.92 (ArC), 125.65 (ArC), 121.91 (ArC), 114.92 (ArC), 104.86 (ArC), 96.50 (Cspiro) ppm. ESI-MS m/z: 422.1 [M+H]^+. 

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4.2.5 Synthesis of 6-chloro-3',5'-diphenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (32c)

Following the general procedure, 190.6 mg of 29a (0.826 mmol, 2 eq) was added to 78.8 mg of 6-Cl isatin (0.413 mmol, 1 eq) which was dissolved in DCM (3 mL). Closed round bottom flask and put the Nitrogen gas balloon. Then, 0.112 mL of TEA (0.826 mmol, 2 eq) was added. Synthesized according to the general procedure, compound 32c was obtained as a light yellow solid in a 54% yield. Reaction time: 18h.

**MP** 239 – 241°C; **IR** ν_{max} (KBr, selected peaks): 3179 (NH), 1737 (C=O), 1598 (C=N), 1493, 1452, 1366, 1287, 1130, 1069, 748, 685, 668 cm\(^{-1}\); **\(^1\)H NMR** (300 MHz, Acetone-d\(_6\)) δ 10.10 (br-s, 1H, NH), 7.88 (dd, \(J = 3.7, 3.1, 1.5\) Hz, 2H, H-A\(_{2,6}\)), 7.57 – 7.50 (m, 4H, H-5, H-A\(_{3,4,5}\)), 7.21 – 7.14 (m, 4H, H-B\(_{3,5}\), H-4, H-7), 6.93 – 6.88 (m, 2H, H-B\(_{2,6}\)), 6.88 – 6.80 (m, 1H, H-B\(_A\)) ppm.; **\(^13\)C NMR** δ (75 MHz, Acetone-d\(_6\)) δ 171.84 (C=O), 152.77 (C=N), 145.02 (ArCq-B), 143.28 (ArCq-7a), 138.61 (ArC), 131.72 (ArC), 130.02 (ArC), 129.72 (ArC), 128.45 (ArCq-A), 127.00 (ArC), 125.74 (ArC), 124.26 (Cq-3a), 123.44 (ArC), 121.85 (ArC), 114.94 (ArC), 112.79 (ArC), 95.56 (Cspiro) ppm.

ESI-MS m/z: 376.2 [M+H]\(^+\).

4.2.6 Synthesis of 7-chloro-3',5'-diphenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one (32d)

Following the general procedure, 190.6 mg of 29a (0.826 mmol, 2 eq) was added to 75.5 mg of 7-Cl isatin (0.413 mmol, 1 eq) which was dissolved in DCM (3 mL). Closed round bottom flask and put the Nitrogen gas balloon. Then, 0.112 mL of TEA (0.826 mmol, 2 eq) was added. For this compound column was repeated with flash chromatography. Synthesized according to the general procedure, compound 32d was obtained as a light yellow solid in a 57% yield. Reaction time: 16h.
4.2.7 Synthesis of 3',5'-diphenyl-3'H-spiro[indole-3,2'-[1,3,4]oxadiazol]-2-one (32e)

Following the general procedure, 235.19 mg of 29a (0.826 mmol, 2 eq) was added to 75.8 mg of isatine (0.51 mmol, 1 eq.) which was dissolved in DCM (3 mL). Closed round bottom flask and put the Nitrogen gas balloon. Then, 0.138 mL of TEA (0.826 mmol, 2 eq.) was added. For this compound column was repeated with flash chromatography. Synthesized according to the general procedure, compound 32e was obtained as a light yellow solid in a 90% yield. Reaction time: 16h.

**MP** 235-237°C; **IR** \(\nu_{\text{max}}\) (KBr, selected peaks): 3151 (NH), 1733 (C=O), 1598 (C=N), 1493, 1472, 1366, 1272, 1128, 746, 683 cm\(^{-1}\), **\(^1\)H NMR** (300 MHz, Acetone-\(d_6\)) \(\delta\) 9.95 (br s, 1H, NH), 7.93 – 7.86 (m, 2H, H-A\(_{2,6}\)), 7.57 – 7.45 (m, 5H, H-A\(_{3,4,5}\), H-5, H-6), 7.21 – 7.09 (m, 4H, H-B\(_{3,5}\), H-4, H-7), 6.94 – 6.88 (m, 2H, H-B\(_{2,6}\)), 6.83 (ddd, \(J = 8.4, 2.2, 1.1\) Hz, 1H, H-B\(_4\)) ppm; **\(^{13}\)C NMR** \(\delta\) (75 MHz, Acetone-\(d_6\)) \(\delta\) 171.88 (C=O), 152.76 (C=N), 143.63 (ArCq-B), 143.41 (ArCq-7a), 133.56 (ArC), 131.63 (ArC), 129.92 (ArC), 129.70 (ArC), 127.00 (ArCq-A), 126.97 (ArC), 125.91 (ArC), 125.77 (ArC), 124.33 (Cq-3a), 124.33 (ArC), 121.55 (ArC), 114.76 (ArC), 112.26 (ArC), 96.04 (Cspiro) ppm. ESI-MS m/z: 342.2 [M+H]\(^+\).
4.2.8 Synthesis of 5-bromo-3',5-diphenyl-3'H-spiro[indole-3,2'-[1,3,4]oxadiazol]-2-one (32f)

Following the general procedure, 153.2 mg of 29a (0.826 mmol, 2 eq) was added to 74.9 mg of 5-Br isatine (0.332 mmol, 1 eq.) which was dissolved in DCM (3 mL). Closed round bottom flask and put the Nitrogen gas balloon. Then, 0.09 mL of TEA (0.664 mmol, 2 eq.) was added. For this compound column was repeated with flash chromatography. Synthesized according to the general procedure, compound 32f was obtained as a dark yellow solid in a 57% yield. Reaction time: 17h.

**MP** 236 - 238°C; **^1H NMR** (300 MHz, Acetone-d$_6$) $\delta$ 10.08 (br s, 1H, NH), 7.92 – 7.86 (m, 2H, H-A$_{2,6}$), 7.75 (d, $J = 2.1$ Hz, 1H, H-4), 7.67 (dd, $J = 8.3$, 2.1 Hz, 1H, H-6), 7.56 – 7.48 (m, 3H, H-A$_{3,4,5}$), 7.23 – 7.15 (m, 2H, H-B$_{3,5}$), 7.13 (d, $J = 8.3$ Hz, 1H, H-7), 6.94 – 6.88 (m, 2H, H-B$_{2,6}$), 6.88 – 6.82 (m, 1H, H-B$_4$) ppm; **$^{13}$C NMR** $\delta$ (75 MHz, Acetone-d$_6$) $\delta$ 171.48 (C=O), 152.75 (C=N), 143.17 (ArCq-B), 142.83 (ArCq-7a), 139.37 (ArC), 131.72 (ArC), 130.05 (ArC), 129.94 (ArC), 129.70 (ArCq-A), 127.04 (ArC), 126.94 (Cq-3a), 125.72 (ArC), 121.85 (ArC), 114.83 (ArC), 114.31 (ArC), 95.72 (Cspiro) ppm. ESI-MS m/z: 422.1 [M+H]$^+$. 

4.2.9 Synthesis of 6-chloro-5'-(4-methoxyphenyl)-3'-phenyl-3'H-spiro[indole-3,2'-[1,3,4]oxadiazol]-2-one (32g)

Following the general procedure, 215.38 mg of 29c (0.826 mmol, 2 eq) was added to 74.9 mg of 6-Cl isatine (0.332 mmol, 1 eq.) which was dissolved in DCM (3 mL). Closed round bottom flask and put the Nitrogen gas balloon. Then, 0.112 mL of TEA (0.826 mmol, 2 eq.) was added. Synthesized according to the general procedure, compound 32g was obtained as a light yellow solid in a 67% yield. Reaction time: 48h.
MP 159 - 162°C, $^1$H NMR (300 MHz, Acetone-d$_6$) $\delta$
10.10 (br-s, 1H, NH), 7.88 (ddd, $J = 3.7, 3.1, 1.5$ Hz, 2H, H-A$_{2,6}$), 7.57 – 7.50 (m, 4H, H-5, H-A$_{3,4,5}$), 7.21 – 7.14 (m, 4H, H-B$_{3,5}$, H-4, H-7), 6.93 – 6.88 (m, 2H, H-B$_{2,6}$), 6.88 – 6.80 (m, 1H, H-B$_4$) ppm;
ESI-MS m/z: 485.3 [M+H]$^+$.  

4.2.10 4.2.3 Synthesis of 6-chloro-3',5'-diphenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one(32h)

Following the general procedure, a solution of a 3-chloro-N'(3-chlorophenyl) benzohydrazonyl chloride (247.49 mg) in DCM (3 ml) was added to 6-chloro isatine (75.0 mg) and TEA (112.06 µL) was added in a dropwise manner. Synthesized according to the general procedure, compound 32h was obtained as a light yellow solid in a 65% yield. Reaction time: 22h.

MP 232 - 234°C; $^1$H NMR (300 MHz, Acetone-d$_6$) $\delta$
10.08 (br s, 1H, NH), 7.92 – 7.86 (m, 2H, H-A$_{2,6}$), 7.75 (d, $J = 2.1$ Hz, 1H, H-4), 7.67 (dd, $J = 8.3, 2.1$ Hz, 1H, H-6), 7.56 – 7.48 (m, 3H, H-A$_{3,4,5}$), 7.23 – 7.15 (m, 2H, H-B$_{3,5}$), 7.13 (d, $J = 8.3$ Hz, 1H, H-7), 6.94 – 6.88 (m, 2H, H-B$_{2,6}$), 6.88 – 6.82 (m, 1H, H-B$_4$) ppm;

4.2.11 Synthesis of 5-bromo-5'-(3-chlorophenyl),3'-(3-chlorophenyl)-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one(32i)

Following the general procedure, a solution of a 3-chloro-N'(3-chlorophenyl) benzohydrazonoyl chloride (198.81 mg) in DCM (3 ml) was added to 5-bromo isatine (75.0 mg) and TEA (90.02 µL) was added in a dropwise manner. Synthesized according
to the general procedure, compound 32h was obtained as a light yellow solid in a 58% yield. Reaction time: 19h.

**MP** 235-236°C; **IR** $\nu_{\text{max}}$ (KBr, selected peaks): 3418 (NH), 1744 (C=O), 1593 (C=N), 1485, 1472, 1209 cm$^{-1}$; $^{1}$H NMR (300 MHz, Acetone-$d_6$) $\delta$ 10.08 (br s, 1H, NH), 7.92 – 7.86 (m, 2H, H-A$_{2,6}$), 7.75 (d, $J = 2.1$ Hz, 1H, H-4), 7.67 (dd, $J = 8.3$, 2.1 Hz, 1H, H-6), 7.56 – 7.48 (m, 3H, H-A$_{3,4,5}$), 7.23 – 7.15 (m, 2H, H-B$_{3,5}$), 7.13 (d, $J = 8.3$ Hz, 1H, H-7), 6.94 – 6.88 (m, 2H, H-B$_{2,6}$), 6.88 – 6.82 (m, 1H, H-B$_{4}$) ppm; $^{13}$C NMR $\delta$ (75 MHz, Acetone-$d_6$) $\delta$ 170.83 (C=O), 152.25 (C=N), 143.96 (ArCq-B), 142.84 (ArCq-7a), 136.82 (ArC), 135.54 (ArC), 135.25 (ArC), 131.84 (ArC), 131.64 (ArC), 130.19 (ArCq-A), 127.32 (ArC), 126.81 (ArC), 126.00 (Cq-3a), 125.64 (ArC), 121.72 (ArC), 116.46 (ArC), 114.92 (ArC), 114.51 (ArC), 112.57 (ArC), 95.70 (Cspiro) ppm.

ESI-MS m/z: 490.94 [M+H]$^+$. 

### 4.2.12 Synthesis of 5-chloro-5'-[3-chlorophenyl],3'-[3-chlorophenyl]-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one(32j)

Following the general procedure, a solution of a 3-chloro-N'-[3-chlorophenyl]benzohydrazonoyl chloride (247.49 mg) in DCM (3 ml) was added to 5-chloro isatine (75.0 mg) and TEA (112.06 $\mu$L) was added in a dropwise manner. Synthesized according to the general procedure, compound 32j was obtained as a light yellow solid in a 75% yield. Reaction time: 19h.

**MP** 234-235°C; **IR** $\nu_{\text{max}}$ (KBr, selected peaks): 3418 (NH), 1744 (C=O), 1593 (C=N), 1485, 1431, 1209 cm$^{-1}$; $^{1}$H NMR (300 MHz, Acetone-$d_6$) $\delta$ 10.08 (br s, 1H, NH), 7.92 – 7.86 (m, 2H, H-A$_{2,6}$), 7.75 (d, $J = 2.1$ Hz, 1H, H-4), 7.67 (dd, $J = 8.3$, 2.1 Hz, 1H, H-6), 7.56 – 7.48 (m, 3H, H-A$_{3,4,5}$), 7.23 – 7.15 (m, 2H, H-B$_{3,5}$),
7.13 (d, J = 8.3 Hz, 1H, H-7), 6.94 – 6.88 (m, 2H, H-B_{2,6}), 6.88 – 6.82 (m, 1H, H-B_{4}) ppm; $^{13}$C NMR δ (75 MHz, Acetone-d$_6$) δ 170.83 (C=O), 152.25 (C=N), 143.98 (ArCq-B), 142.38 (ArCq-7a), 135.53 (ArC), 135.25 (ArC), 133.91 (ArC), 131.84 (ArC), 131.63 (ArC), 129.35 (ArCq-A), 127.40 (ArC), 127.31 (ArC), 126.81 (Cq-3a), 127.31 (ArC), 125.64 (ArC), 121.72 (ArC), 114.93 (ArC), 114.09 (ArC), 112.60 (ArC), 95.77 (Cspiro) ppm.

ESI-MS m/z: 445.0 [M+H]$^+$. 

### 4.2.13 Synthesis of 5-chloro-5'-(4-chlorophenyl)-3'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]oxadiazol]-2-one(32k)

Following the general procedure, a solution of a $N'$-(4-chlorophenyl)benzohydrazonoyl chloride (219.03 mg) in DCM (4.13 ml) was added to 5-chloro isatine (75.0 mg) and TEA (112.06 µL) was added in a dropwise manner. Synthesized according to the general procedure, compound 32k was obtained as a light yellow solid in a 69% yield. Reaction time: 16h.

**MP** 237-239°C; **IR** $\nu_{\text{max}}$ (KBr, selected peaks): 3369 (NH), 1763 (C=O), 1593 (C=N), 1493, 1474, 1395, 1242, 1194, 764, 690 cm$^{-1}$; **$^1$H NMR** (300 MHz, Acetone-d$_6$) δ 10.08 (br s, 1H, NH), 7.92 – 7.86 (m, 2H, H-A$_{2,6}$), 7.75 (d, $J = 2.1$ Hz, 1H, H-4), 7.67 (dd, $J = 8.3$, 2.1 Hz, 1H, H-6), 7.56 – 7.48 (m, 3H, H-A$_{3,4,5}$), 7.23 – 7.15 (m, 2H, H-B$_{2,5}$), 7.13 (d, $J = 8.3$ Hz, 1H, H-7), 6.94 – 6.88 (m, 2H, H-B$_{2,6}$), 6.88 – 6.82 (m, 1H, H-B$_{4}$) ppm; **$^{13}$C NMR** δ (75 MHz, Acetone-d$_6$) δ 171.29 (C=O), 153.24 (C=N), 142.39 (ArCq-B), 142.06 (ArCq-7a), 133.66 (ArC), 131.94 (ArC), 129.74 (ArC), 129.49 (ArC), 129.39 (ArC), 129.21 (ArCq-A), 128.19 (ArC), 127.23 (ArC), 127.16 (Cq-3a), 126.26 (ArC), 126.10 (ArC), 125.48 (ArC), 116.35 (ArC), 114.04 (ArC), 95.70 (Cspiro) ppm.

4.2.14 Synthesis of 5-bromo-5’-(4-chlorophenyl)-3’-phenyl-3’H-spiro[indoline-3,2’-[1,3,4]oxadiazol]-2-one (32l)

Following the general procedure, a solution of a N’-(4-chlorophenyl)benzohydrazonoyl chloride (175.96 mg) in DCM (3.31 ml) was added to 5-bromo isatin (75.0 mg) and TEA (90.02 µL) was added in a dropwise manner. Synthesized according to the general procedure, compound 32l was obtained as a light yellow solid with 66% yield. Reaction time: 16h.

**MP** 243 - 245ºC; ¹H NMR (300 MHz, Acetone-d₆) δ 10.08 (br s, 1H, NH), 7.92 – 7.86 (m, 2H, H-A₂,₆), 7.75 (d, J = 2.1 Hz, 1H, H-4), 7.67 (dd, J = 8.3, 2.1 Hz, 1H, H-6), 7.56 – 7.48 (m, 3H, H-A₃,₄,₅), 7.23 – 7.15 (m, 2H, H-B₃,₅), 7.13 (d, J = 8.3 Hz, 1H, H-7), 6.94 – 6.88 (m, 2H, H-B₂,₆), 6.88 – 6.82 (m, 1H, H-B₄) ppm; ¹³C NMR δ (75 MHz, Acetone-d₆) δ 171.36 (C=O), 155.14 (C=N), 149.62 (ArCq-B), 141.20 (ArCq-7a), 134.01 (ArC), 132.64 (ArC), 129.48 (ArC), 129.39 (ArC), 128.20 (ArCq-A), 127.93 (Cq-3a), 124.50 (ArC), 120.55 (ArC), 115.76 (ArC), 115.36 (ArC), 115.22 (ArC), 95.70 (Cspiro) ppm.

4.3 Biological Experiments

HCT116 cells were grown in McCoy’s 5A supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA), 1% GlutaMAX™ (Invitrogen) and 1% penicillin/streptomycin solution (Sigma-Aldrich, St Louis, MO, USA). SW620 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS and 1% antibiotic/antimycotic solution (Sigma-Aldrich). For cell viability assays cells were seeded at 1 x 10^5 cells/mL (HCT116 and SW620); (HCT116, SW620) and for flow cytometry analysis cells were seeded at 3 x 10^5 cells/mL. HCT116 human colorectal carcinoma cells rendered p53-null by somatic knockout were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD).

**In vitro cytotoxicity assay**

The cellular growth inhibitory activity was evaluated in four cell lines: an isogenic matched pair of wild type p53 and deleted human colorectal cancer cell lines [HCT116 p53^{+/+} and p53^{-/-}], human colorectal adenocarcinoma cell lines [SW620 (mut p53)] and normal colon fibroblasts (CCD-18Co).

HCT116, SW620 cells were incubated with vehicle or the compounds approximately 24 h after plating. For the assays with these cell lines, the compounds were dissolved in DMSO and diluted in culture medium to a range of concentrations from 0.5 to 200 μM (at least twelve concentrations were used). CCD-18Co cells were grown in DMEM supplemented with 10% FBS; 1% Pen/Strep; 1% Glutamate; 1% Non-essential amino acids (NEAA); 0.025% TNF-alpha Human. The final concentration of DMSO in culture medium during treatment did not exceed 0.8% (v/v) and the same concentration of DMSO was added to the control. Each compound concentration and DMSO was tested in duplicate in a single experiment that was repeated 3 times. Cells were incubated at 37 ºC in humidified 5% CO₂ atmosphere. Cell viability was assessed 72 h after compound incubation by using the CellTiter96® AQueous Non-Radioactive Cell Proliferation Assay (Promega), according to the manufacturer’s protocol. The method is based on the reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. The
absorbance was measured at 490 nm using Bio-Rad microplate reader Model 680 (Bio-Rad, Hercules, CA, USA).

The concentrations of the compounds that inhibited cell growth by 50% (IC50) were determined by non-linear regression using GraphPad PRISM software.
CHAPTER IV – REFERENCES
REFERENCES


(63) Turbiak, A.; Showalter, H. A New Route to Substituted Pyrimido[5,4-E]-1,2,4-Triazine-5,7(1H,6H)-Diones and Facile Extension to 5,7(6H,8H) Isomers. *Synthesis (Stuttg)*. 2009, 2009 (23), 4022–4026.


(70) Raunak; Kumar, V.; Mukherjee, S.; Poonam; Prasad, A. K.; Olsen, C. E.; Schäffer, S. J. C.; Sharma, S. K.; Watterson, A. C.; Errington, W.; Parmar, V. S.


A.1. NMR and Mass Spectra

A.1.1 Compound (32a)

A.1.1.1. $^1$H NMR

A.1.1.2. COSY
A.1.1.3. APT

A.1.1.4. HMQC
A.1.2. Compound (32b)

A.1.2.1. $^1$H NMR

A.1.2.2. COSY
A.1.2.3. APT

A.1.2.4. HMQC
A.1.2.5. HMBC

A.1.2.6. MS
Compound (32e)

A.1.5.1. $^1$H NMR

A.1.5.2. COSY
A.1.5.3. APT

A.1.5.4. HMQC
Compound (32f)

A.1.6.1. $^1$H NMR

A.1.6.2. COSY
A.1.6.3. APT

A.1.6.4. HMQC
A.1.6.5. HMBC

A.1.6.6. MS