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Novel Trisubstituted Arylidene Oxindoles with Potent Anti-Apoptotic Properties

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NOVEL TRISUBSTITUTED ARYLIDENE OXINDOLES WITH POTENT
ANTII-APOPTOTIC PROPERTIES

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

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B.S., Wright State University, 2009

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ABSTRACT


Apoptosis is a highly complex, multi-step cellular pathway utilized to initiate cell suicide. The family of enzymes largely responsible for the process is the cysteinyl aspartic acid proteases (caspases). This study details the design and synthesis of a new class of small-molecule caspase inhibitors, namely arylidene oxindoles, which are comparable to the isatin family of inhibitors but which offer the advantage of incorporating three points of variability into the basic scaffold. Moreover, whereas the mode of action of isatins against caspases has been demonstrated to involve nucleophilic addition of the cysteine residue of the enzymes to the C-3 ketone carbonyl of these substrates, the efficacy of arylidene oxindoles might be expected to involve a Michael attack of the cysteine sulfur onto the beta-carbon of the heterocyclic system in a Michael fashion. Furthermore, NMR investigations into the E/Z isomerization of such arylidene oxindoles were conducted which show that with few exceptions, isomerization is occurring in polar solvents, in which case it was deemed unnecessary to chromatographically separate isomers prior to screening. Finally, the anti-apoptotic properties of the designed molecules were examined with Human Jurkat T lymphoma cell apoptosis in a DNA Ladder assay.
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Thank you.
1. INTRODUCTION TO OXINDOLE CHEMISTRY

Oxindole (1, with accepting numbering system shown) was first synthesized in the late 19th century by Baeyer as a result of the synthetic reduction of isatin (2). Oxindole needles are colorless and odorless with a melting point of 126°C and readily dissolve in hot water and organic solvents such as acetic acid and alcohols as well as benzene, acetone and dimethylsulfoxide. Oxindole-hydrochloride salts can be readily formed by reaction with hydrochloric acid and thus become freely soluble in water.

\[
\text{[O]} \quad \text{[H]}
\]

\[
\overset{\text{N}}{\begin{array}{c}
\text{N} \\
\text{H}
\end{array}} \quad \overset{\text{O}}{\begin{array}{c}
\text{O}
\end{array}}
\]

NMR studies by Gassman have determined explicit delta (\(\delta\)) values for the carbon atoms within the ring of oxindole. The NMR samples were run in DMSO (DMSO-\(d_6\)) at room temperature, and the \(^{13}\text{C}\) \(\delta\) values were calculated relative to trimethylsilane (TMS). From this study, the following assignments were made: \(C_2: \delta 176.6\) ppm; \(C_3: \delta 36.3\) ppm; \(C_{3a}: \delta 125.4\) ppm; \(C_4: \delta 124.4\) ppm; \(C_5: \delta 122.2\) ppm; \(C_6: \delta 127.5\) ppm; \(C_7: \delta 109.3\) ppm; \(C_{7a}: \delta 143.8\) ppm.

The chemistry of oxindoles prior to 1945 was reviewed by Sumpter and includes both syntheses and reactions of this heterocycle. A summary of this chemistry including more recent findings will be provided herein with the synthetic approaches being categorized using the concept of bond disconnection to specify the bonds formed in the synthesis (Scheme 1).
As stated earlier, the first reported synthesis of oxindole was by Baeyer and involved the reduction of isatin (2) in a two step process using sodium amalgam and base to initially form an intermediate 3-hydroxy-indolinone 3. This hydroxyl indolinone was then further reduced under acidic conditions in the presence of tin to afford oxindole (1).

The reduction of the carbonyl groups of aldehydes and ketones to a methylene group is most commonly effected by means of the Wolff-Kishner reduction involving formation and decomposition of the corresponding hydrazones in the presence of sodium ethoxide at 180-200°C for 6-8 h. The microwave assisted version of this procedure was later described using 55% hydrazine hydrate and ethylene glycol (medium power, 30 s) followed by treatment of the resultant hydrazone with potassium hydroxide in ethylene glycol for 10 seconds.
Noting that the hydrazones of α-dicarbonyl compounds may be decomposed under relatively mild conditions, Crestini devised a one-pot Wolff-Kishner reduction of isatins by refluxing the isatins 2 in 98% hydrazine hydrate for 15-30 min without isolation of the intermediate hydrazones 4.\(^5\) The authors attribute the facility of this process in the absence of additional base to the possibility of the α-ketoamide to give anchimeric assistance in the stage of decomposition of the hydrazone.

![Chemical structure](image)

**B. NITROGEN-C2 BOND FORMATION**

The structure of oxindole was initially established by Baeyer who was also the first to report a ring forming synthesis based on the cyclization of o-nitrophenylacetic acid (5). In this process, reduction (Sn/HCl) of the nitro group of 5 to the aniline 6 was followed by acid promoted cyclization to oxindole.\(^2\)

![Chemical structure](image)

In an alternate version of this general protocol, o-chlorophenylacetic acid 7 was treated with ammonium hydroxide in the presence of cupric acetate and heated in a sealed pressure tube (160°C, 8 h) to presumably form the amine intermediate 6 which then cyclized to oxindole.\(^7\)
Later variants of this process focused on alternate means of constructing the phenylacetic acid moiety. For instance, in one synthesis of 4-alkoxyoxindoles, the ortho-hydroxylated nitrotoluene 8 was O-alkylated with alkyl halides after treatment with NaOMe, and the resultant ethers 9 were condensed with diethyl oxalate (DEO) and KOEt to produce an intermediate 10, which was not isolated. Treatment of intermediate 10 with peroxides then afforded the o-nitrophenylacetic acids 11. Cyclization was performed under reductive conditions by treatment of the phenylacetic acids with H₂/Pd-C to yield the cyclized products 12.⁸

In another approach, Quallic and Morrissey⁹ utilized a sequence involving initial nucleophilic aromatic substitution of o-halonitrobenzenes 13 (X= -Cl, -F, -Br. R = -Cl, -F, -OMe). Thus, SNAr-type reaction of the halonitrobenzenes with dimethyl malonate
(DMM) in the presence of NaH in DMSO afforded the ortho-alkylated derivatives 14 which were then decarboxylated by addition of LiCl in DMSO and water at 100°C for 3 hours. The resulting phenylacetic acids were then cyclized in the presence of Fe/AcOH to the substituted oxindoles 15.\(^9\)

![Chemical diagram](image)

Gassman utilized aniline derivatives in what is perhaps the most general synthesis of oxindoles.\(^10\) In this procedure, conversion of anilines 16 to the ortho-substituted anilines 19 was accomplished by treating the aniline with tert-butylhypochlorite (tBuOCl) in dichloromethane at low temperature to afford N-chloro anilines 17. Addition of sulfides such as ethyl 2-(methylthio)acetate then led to the azasulfonium salt intermediates 18. These non-isolated intermediates then undergo deprotonation of the alpha carbon forming C-S ylides which undergo subsequent Sommelet-Hauser rearrangement to afford the ortho-ethyl-2-(2-aminophenyl)-2-(methylthio)acetates 19. Cyclization was performed under dilute acidic conditions to produce the thio-substituted oxindoles 20, which upon reductive desulfurization with Raney nickel affords the oxindoles 21. In addition, the use of substituted aniline derivatives allows for the incorporation of 5-possition substituents (X = -NO\(_2\), -CH\(_3\), -CO\(_2\)C\(_2\)H\(_5\)) directly into the oxindole ring, rather than being introduced by post-synthesis modifications.
Although numerous biologically active indoles and oxindoles bear methoxyl functions in the 5-, 6-, and 7-positions of the heterocyclic nuclei, it was disappointing that p-anisidine gave ca. 2% of the ortho rearranged aniline due to the instability of the corresponding N-chloro intermediate.

Most recently a simplified version of the Gassman synthesis was reported involving the reaction of α-diazocarbonyl compounds and sulfenamides 22. In this protocol, rhodium catalyzed decomposition of the α-diazocarbonyl compound leads to a carbene which reacts with the sulfenamide to generate a sulfonium ylide 23 directly. The ylide then undergoes spontaneous thia Sommelet-Hauser rearrangement to the ortho-substituted aromatic compounds 24 followed by cyclization to give polysubstituted oxindoles 25. The authors note that the Rh-catalyzed decomposition of the diazocarbonyl circumvents the steps normally needed in the Gassman protocol to generate the ylide, namely: a) generation of the N-chloroaniline, b) formation of an azasulfonium salt, and c) ylide generation by treatment with base.
C. C3 – C3a BOND FORMATION

As reported in the review by Sumpter, Stolle reported the first intramolecular Friedel-Crafts reactions to produce oxindole, much of which appeared in German patents. This involved the reaction of aniline (26) in the presence of chloroacetyl chloride to produce alpha-chlorophenylacetamide (27) which was subsequently treated with AlCl₃ in benzene at 160°C to produce oxindole.

Daisley and coworkers at the University of London developed a modification of the Stolle reaction, wherein N-substituted oxindoles were prepared by first acylating aniline (28) with acetic anhydride to form N-phenylacetamide (29). Treating 29 with NaH in the presence of an alkyl halide then led to the N-alkylated amides 30. Acidic deacylation to form 31 was accomplished in the presence of sulfuric acid after which the N-alkylated anilines were reacylated with 2-chloroacetyl chloride to form the tertiary amides 32. Cyclization was performed via intramolecular Friedel-Crafts reaction with anhydrous AlCl₃ at 160°C to produce N-substituted oxindoles 33.
In 1972, R.F. Heck reported that aryl, benzyl and styryl halides react with olefinic compounds in the presence of a hindered amine and a catalytic amount of palladium acetate to form vinylic derivatives in which the aryl, benzyl, or styryl group has replaced a vinylic hydrogen of the original. The important part of this reaction is that the resultant product had retained the double bond while substituting the hydrogen with the aryl group. This reaction still remains one of the prominent routes to such substituted double bonds. The reaction requires use of ligated Pd(II), generally Pd(OAc)$_2$ to react with a halogen-containing compound and the hydrogen on a double bond. The hindered amine acts as a base and the Pd catalyzes the reaction so that double bond configuration is retained (Scheme 2).

Scheme 2 – R. F. Heck Vinyl substitution

Heck subsequently demonstrated the palladium-catalyzed intramolecular cyclization of N-acryloyl-o-bromoanilines as a route to oxindoles. Thus,
cyclization of both N-cinnamoyl- (R = H) and N-(β-methylcinnamoyl)-β-bromoaniline (R = CH₃) at 100 °C with 1 mol % palladium acetate and 4 mol % of tri-β-tolylphosphine led to formation of the oxindole derivatives.¹⁶

\[
\begin{align*}
\text{34} & \quad \text{Pd(OAc)}_2, P(\text{o-tol})_3 \quad 100 \, ^\circ \text{C}, \text{Et}_3\text{N} \\
\text{35}
\end{align*}
\]

A unique application of the Heck reaction was the synthesis of oxindole in an intramolecular fashion on solid support.¹⁶ In this reaction, a p-alkoxy iodoaniline is anchored to the Rink amide resin via coupling of the carboxyl derivative of the aniline with DCC (not shown) to give compound 36. The amine is reductively alkylated with aldehydes in the presence of NaBH(OAc)$_3$ to afford secondary amines 37 then subsequent acylation with an alpha-beta unsaturated acid chlorides gives the tertiary aniline derivatives 38. The Heck reaction is then employed with Pd(OAc)$_2$ catalyst to give the oxindole 39 after removal from the resin with TFA in DCM. The final product 39 was shown to form in both $E/Z$ isomers, predominantly $E$ stereochemistry after isolation/removal from the resin. The ratio of $E/Z$ isomers was determined by HPLC and the authors report no further stereochemical analysis.¹⁶
In reference to newly designed metal-assisted cyclizations, Buchwald\textsuperscript{17} introduced a modified Friedel-Crafts type reaction involving Pd-coordinated oxindole synthesis from alpha-chlorophenylacetamides in the presence of TEA and 2-(di-tert-butylphosphino)biphenyl ligand. The proposed mechanism is, initial treatment of the chloro-phenylacetamide \(40\) with palladium resulting in a theoretical Pd(II) complex \(41\) generated from the transfer of the electrons followed by intramolecular electrophilic substitution \textit{ortho} to the amide based on the Friedel-Crafts method followed by reductive elimination of the Pd complex to give oxindole \(42\). Another proposed mechanism by Buchwald uses the same C-H activation through coordination of Pd enolate with the quaternary carbon P-orbital in an orbital interaction followed by reductive elimination to afford the oxindole.\textsuperscript{17}

A number of recent approaches to the synthesis of oxindoles have relied upon the development of the cyclization of anilides involving metal catalysts.\textsuperscript{18,19} One author presented it as a direct C-H to Ar-H coupling.\textsuperscript{18} This reaction involves a tertiary aniline, such as \(43\) in the presence of Pd(OAc)\(_2\)/Cu(OAc)\(_2\) with potassium tert-butoxide. This
mixture is promoting the coordination of 43 with Pd(II) after base abstraction of the proton and Cu(II)-mediated generation of a radical. The Pd complex then couples to the benzene ring ortho to the location of the amide to form the intermediate 44. The reaction proceeds through reductive elimination by reduction of Pd(II) to Pd(0) and formation of the Ar-C bond to complete the formation of the oxindoles 45.

Preparation of oxindoles from the previously proposed scheme relies on N-alkylated amide-linked electron withdrawing substituents on the alpha-carbon such as the ethyl ester. Murakami, et al. introduces the synthesis as a carbon-carbon coupling reaction, which presents another route to substituted oxindoles via metal catalysis. This process occurs in four steps: coordination, followed by oxidative cyclization involving the Pd catalyst, then a ligand substitution, with subsequent removal of Pd in a reductive elimination. For example, an alkynyl-aryl isocyanate 46 is treated with trifluoroacetamide 47 in the presence of Pd$_2$dba$_3$-CHCl$_3$/dppf (dppf = 1,1’-bis(diphenylphosphino)ferrocene), dba = dibenzylideneacetone) to promote intramolecular coordination of Pd(0) between the isocyanate and alkyne group 48a. Oxidative cyclization occurs through formation of a Pd(II) complex 48b and then alkyl substitution intermediately occurs at the Pd(II) bond using 47 (trifluoroacetamide) as the alkylating agent followed by reductive elimination of Pd(II) to form the highly
substituted oxindole 49.

\[
\begin{array}{c}
\text{46} + \text{47} \rightarrow \text{48a} \rightarrow \text{48b} \rightarrow \text{49}
\end{array}
\]

D. SYNTHETIC MODIFICATIONS

**Halogenation, Nitration, Sulfonation**

Baeyer was the first to demonstrate the selective functionalization of the oxindole ring in addition to being the first to synthesize this heterocycle. Baeyer’s method of C-5 nitration involved potassium nitrate and sulfuric acid\(^2\). Prior to 1945, other halogenation, nitration and sulfonation reactions were employed on oxindole without firm establishment of the correct substitution patterns.\(^3\) Sumpter eventually firmly established that the position and number of substitutions in the halogenation of oxindole is dependent on solvent and molar equivalents of halogen\(^{20}\) (Scheme 3).
Scheme 3 – Sumpter’s evaluation of the bromination of oxindole

Sumpter found that in the case of bromination, addition of one equivalent of bromine in water affords the 5-bromo oxindole (i, above), whereas 2 equivalents of bromine in water gives 5,7-dibromo oxindole (ii). Treatment of the 5-bromooxindole with 2 equivalents of bromine in carbon tetrachloride yields 3,3,5-tribromo oxindole (iii) while treatment of 5,7-dibromo oxindole with 2 equivalents of bromine in carbon tetrachloride yields 3,3,5,7-tetrabromo oxindole (iv). Addition of 3 equivalents of bromine in water to oxindole gives 3,5,7-tribromo oxindole. Alternatively oxindole treated with just 2 equivalents of bromine in carbon tetrachloride yields 3,3-dibromooxindole (v). More recently, it has been shown that bromination can be accomplished by treating oxindole 1 with bromine in a warm 90°C solution of Br₂/KBr and water to give 5-bromo oxindole 50.
Also reported for bromination of oxindole 1 is the treatment with N-bromosuccinimide at low temperature in acetonitrile to afford 5-bromo oxindole 50.\(^{22}\)

\[
\text{\begin{align*}
\text{NBS} & \quad \xrightarrow{\text{ACN, 0°C}} \\
\begin{array}{c}
\text{1} \\
\text{Br} \\
\text{N} \\
\text{O}
\end{array} & \quad \begin{array}{c}
\text{50} \\
\text{N} \\
\text{O}
\end{array}
\end{align*}
\]

A method has been reported for the preparation of 5-chloro-oxindole\(^{23}\) by treating oxindole in aqueous solution with chlorine gas.

Direct acylation of oxindoles is not widely reported in the literature and most of that is obscurely described in patents. Of the few clearly reported cases, it is found that Friedel-Crafts acylation can be accomplished by treating oxindole 1 with butyryl chloride in the presence of AlCl\(_3\) in DCM\(^{24}\) to give 5-butyryl indolin-2-one (51).

\[
\text{\begin{align*}
\text{AlCl} & \quad \xrightarrow{\text{DCM}} \\
\begin{array}{c}
\text{1} \\
\text{O}
\end{array} & \quad \begin{array}{c}
\text{51} \\
\text{O}
\end{array}
\end{align*}
\]

Also reported by Sumpter was the sulfonation of 3,3-dimethyl oxindole through addition of oxindole to fuming sulfuric acid to produce 3,3-dimethyl-5,7-disulfonate oxindole.\(^3\) Later, formation of a 5-sulfonyl chloride oxindole\(^{25,26}\) derivative was accomplished via reaction of oxindole 1 with chlorosulfonic acid at room temperature followed by warming the reaction mixture to 60°C for 3 h to give the 2-oxoindolin-5-sulfonyl-chloride 52 which is isolated but not characterized. Filtration of this crude sulfonyl chloride and treatment with amines gives the corresponding 5-sulfonamide-oxindoles 53.
Nitration by Baeyer was accomplished using sulfuric acid and potassium nitrate in solution. Although Baeyer had exceptional skill in structural analysis, it was not determined until many years later where the nitration had occurred. Sumpter later verified the substitution site by generation of the beta-oxime of 5-nitro-oxindole after nitro oxindole was reacted with acetic acid in sodium nitrite. This sample yielded an identical melting point to a known 5-nitro-beta-oxime oxindole, which concluded that the nitration of oxindole did occur at the 5-position.  

**N-Alkylation**

N-Alkylation of oxindole cannot be regioselectively achieved by treating this heterocycle with base in the presence of alkyl halides. This limitation is due to the ambident (bifunctional) reactivity of oxindole at the N- and 3-positions, where the majority of alkylation occurs first at the 3 position. This is undesirable unless the requirement is to have the same alkylation substitutions at both the N- and 3-positions. Therefore, to achieve the selective N-alkylation of oxindole, one normally follows a two-step protocol involving N-alkylation of an isatin followed by reduction to the corresponding oxindole. N-Alkylation of isatin is accomplished by treating isatin with base in refluxing acetonitrile. Addition of alkylating agents such as alkyl halides gives the N-alkylated isatin. Subsequent reduction of the carbonyl at the 3-position gives the corresponding N-alkylated oxindole.
A variety of methods exist for the N-alkylation of isatins. Some of the more general methods include the use of calcium hydride (CaH$_2$, 40-50 °C)$^{39,45}$ or sodium hydride (NaH, 25-80 °C) in DMF$^{40,43}$ which has been found suitable for derivatives with electron withdrawing substituents on the aromatic nucleus; or the use of K$_2$CO$_3$ or Cs$_2$CO$_3$ (DMF, 80 °C)$^{44}$. Given the susceptibility of 5-nitroisatin to undergo nucleophilic cleavage of the N1-CO bond under basic conditions, a novel modified protocol was developed for N-alkylation employing a mild base combination of CuCO$_3$/Cs$_2$CO$_3$ (1:2) in anhydrous DMF.$^{41}$

Alternatively, the use of microwave irradiation has been employed to expedite this process and was first reported by Azizian using K$_2$CO$_3$/DMF or NaOEt/EtOH in a conventional microwave oven.$^{42}$ More recently, a comprehensive reinvestigation of this protocol was performed by Perillo $et$ $al.$ who examined a range of bases (Na$_2$CO$_3$, K$_2$CO$_3$, Cs$_2$CO$_3$, CaH$_2$, TEA, LiOH, NMM, NaOEt) in a range of solvents (DMF, DMA, HMPT, MeCN, DMSO, NMP) and found the optimal conditions to consist of K$_2$CO$_3$ or Cs$_2$CO$_3$ and a few drops of N,N-dimethylformamide or N-methyl-2-pyrrolidinone.$^{47}$

Lindsley developed a parallel microwave procedure for the preparation of a variety of N-beznyl isatins employing K$_2$CO$_3$/KI in acetonitrile (CAN) under microwave conditions (160 °C, 10 min) using a Biotage Initiator-60 instrument,$^{48}$ while Go reported an analogous process in DMF (150 °C, 5-15 min) in a similar reactor.

The N-carboxymethylation of isatin using ethyl bromoacetate has likewise been effected under a variety of conditions, including calcium hydride (DMF, 40 °C)$^{39,45}$ and under microwave conditions using K$_2$CO$_3$ or Cs$_2$CO$_3$.$^{47}$ More recently, Martin described a protocol for the preparation of (2,3-dioxo-indol-1-yl)acetamides involving initial N-
carboxyalkylation of isatin using ethyl bromoacetate (1.5 equiv) under basic conditions (1.5 equiv of K$_2$CO$_3$ in DMF, rt, 48 h) in the presence of tetrabutylammonium bromide (TBAB, 0.1 equiv) as the phase transfer catalyst. 46 The ester function was saponified to the corresponding carboxylic acid using sodium hydroxide (2 equiv) for 4 h at room temperature in ethanol/water (1/1) followed by neutralization with aqueous HCl (3 M).

The amides were then prepared by treatment with ethyl chloroformate (1.2 equiv) and triethylamine (2 equiv) for 30 min at -10 °C followed by reaction with the desired amines (2 equiv) for 2h.

**E. C3 FUNCTIONALIZATION**

A method for direct alkylation of oxindoles was reported involving treatment of an ethanolic solution of N-methyl oxindole (54) with ethyl bromoacetate (55) in the presence of sodium ethoxide (rt, 2 h). A oily mixture was reported as product: the oil being a combination of N-methyl oxindole (starting material), and the alkylation product.28 The reported alkylation product of this reaction was N-methyl-3,3-dicarbethoxymethylxindole (56). The oxindole had undergone dialkylation at the C-3 position.28

![Chemical structure](image)

Dialkylation at the 3-position was uncontrollable initially, but further studies showed generation of the enolate of oxindole prior to reaction with the participating alkyl
halide would produce monosubstitution. Monosubstitution occurred after mixing sodium hydride in benzene with 54 to generate the enolate 57. After generation of enolate, the suspension was added to a benzene solution of ethyl bromoacetate producing the monoalkylated oxindole 58.

The condensation of aromatic, heterocyclic, and alkyl aldehydes and ketones with oxindole is widely reported in the literature. The most commonly followed literature protocol generally involves the use of 10 mol % piperidine in ethanol. These arylidene/alkylidene oxindole compounds are reported to exhibit a number of useful biological properties and great efforts have been made to screen these compounds. A reaction by I. W. Elliot, et al. is one of the first reported condensation reactions. These workers condensed oxindole (1) with 2-pyridinecarboxaldehyde (59) in a solution of iso-propyl alcohol and small amounts of piperidine (2 mL) to produce 3-(pyridin-2-ylmethylene)indolin-2-one (60).

A “dry condensation” process under microwave irradiation was reported. This
report described the condensation of oxindole with aromatic aldehydes or heterocyclic aldehydes. Oxindole (1) and the respective reactant aldehyde, in this example, benzaldehyde (61), were initially stirred in acetonitrile and a large excess of KF-Al₂O₃ for five minutes at room temperature. Solvent was evaporated and the solid was irradiated at 2450 MHz followed by extraction with acetonitrile to give condensation product 62.

\[
\text{Oxindole (1)} + \text{benzaldehyde (61)} \xrightarrow{\text{KF-Al}_2\text{O}_3} \text{Product 62}
\]

The oxindoles described herein were developed in order to provide a unique scaffold for development of a small-molecule caspase (apoptosis) inhibitor. Oxindole provides a near-planar core scaffold, the 3-position allows for a greater diversity of molecules in a combinatorial library, the scaffold has a modifiable 5-position and a stable lactam linkage for a proposed α,β-unsaturated Michael acceptor. This Michael acceptor is to be located at the 3-position from condensations of the aryl aldehydes as described above. The beta carbon is proposed to undergo Michael addition by the cysteine sulfur atom of the caspase enzyme (described in the biology introduction). This covalent interaction is proposed to be the main mechanism of the inhibitor.

II. A GENERAL BIOLOGY INTRODUCTION OF CASPASES AND CASPASE INHIBITORS

Apoptosis is a naturally occurring process that triggers a cell to collapse, condense and eventually die. Living organisms need apoptosis to maintain homeostasis.
Overexpression of apoptosis may be responsible for some of the pathologies seen in human disease. The biological mechanism for this process is very complex with multiple pathways. One family of enzymes has been identified as both initiators (proenzymes) and effectors (those carry out the cellular functions) of apoptosis. These enzymes are known as caspases, cysteinyl-aspartic acid proteases. Cysteine residues located within the caspases have specific binding to aspartic acid within a small sequence of amino acids. The sequence that is known to trigger the protease activity is different for each caspase, however the pattern of the relevant tetrapeptide recognition sequence is -X₁-E-X₂-D-. The specific amino acids for X₁ and X₂ can be found in Table 1. Abbreviations common to amino acids are one-letter designations. For example, D is short for aspartic acid, E is short for glutamic acid, V is short for valine, etc. Therefore, if …-aspartic acid-glutamic acid-valine-aspartic acid-… is the cleavage sequence found in caspase-3 then it shall be abbreviated D-E-V-D or DEVD. The cleavage of the target protein specifically at the aspartic acid residue is key for activation and execution of the caspases. The following table (Table 1) summarizes the caspase number designation, the function of the protein and the specificity sequence.

<table>
<thead>
<tr>
<th>Caspase designation (no.)</th>
<th>Biological function</th>
<th>Cleavage sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 4, 5</td>
<td>Inflammatory</td>
<td>(W/Y)-(V/A/E)-(H/A)-D&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3, 6, 7</td>
<td>Repair, homeostasis</td>
<td>D-E-(T/V/H)-D&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2, 8, 9, 10</td>
<td>Proenzyme</td>
<td>(L/V/I)-E-(H/T)-D&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> caspase 1 – YVAD, caspase 4/5 – WEHD <sup>b</sup> caspase -3/7 – DEVD, caspase 6 - VEID <sup>c</sup> caspase 2 (unique) – VDVAD

Following the discovery of the cleavage sequence involving aspartic acid, a number of peptide-based caspase inhibitors were developed. The now-classical inhibitors consist
of an Ac-X-D-R sequence,$^{35}$ where Ac is an acetyl group, basically a nonspecific, neutral end cap for the chain.

![Diagram](image)

**Figure 1** – Schematic of classic peptide caspase inhibitors

In the generalized structure above (**Figure 1**), [X] can be considered a variable peptide sequence of from one to four amino acids in length. For example, if valine, alanine, and glutamic acid are found, a V-A-E sequence resides N-terminally to the aspartic acid residue. The choice of amino acid in this class of inhibitors on the C-terminal side of the aspartic acid has not been shown to confer specificity for given caspases. The critical aspartic acid residue, D, is located adjacent to the active binding site and/or leaving group “R.” The leaving group is responsible for determining the reversible/irreversible nature of the inhibitor. Through complex binding mechanisms not described in this thesis, it has been determined that aldehyde, ketone and nitrile leaving groups are reversible$^{34}$ whereas methyl ketones are irreversible.$^{34}$ Attached to the methyl ketone, depending on inhibitor, are a primary halogen (-Cl, -F), acyloxy-, or diazo-group.

The second generation of peptide inhibitors are the non-acetyl, N-capped peptide inhibitors wherein the generalized acetyl above is replaced by benzyloxy carbonyl (Z), or N-Boc.$^{35}$ These inhibitors are shown to inhibit actinomycin D-induced apoptosis of human jurkat T-cells in a DNA-ladder assay at concentrations starting at 100 µM. A DNA ladder assay is used as a generalized test to see if a therapeutic is capable of preventing apoptosis from being executed. Actinomycin D is a small peptide that
interrupts the DNA repair machinery and triggers the apoptotic pathway. It is not a test designed for any specific enzyme, however the test does show ability to prevent apoptosis as the measurement for generalized therapeutic effectiveness in the lowest concentration possible (potency). The weakest of commonly employed inhibitors is Z-VAD-fmk (Figure 2, fmk = fluoromethyl ketone), which has a poor (>50 µM) inhibitory potential against actinomycin-D induced apoptosis of human jurkat T lymphoma cells. Removing the V-A amino acid linking groups and replacing Z with N-Boc yields Boc-D-fmk (Figure 3), which exhibits a lower inhibition concentration (more potent) in the vicinity of 50 µM. 

![Figure 2 - Z-VAD-fmk](image1)

![Figure 3 - Boc-D-fmk](image2)

Evolution of the peptide-based caspase inhibitors replaced classical peptide blocking groups with hydrophobic heterocycles as found in the highly potent caspase inhibitor Q-VD-OPh (Figure 4).

![Figure 4 - Q-VD-OPh](image3)
Q-VD-OPh is capable of inhibiting apoptosis triggered by actinomycin D at concentrations of 5 µM in DNA ladder assay using human jurkat T lymphoma cells. Its high potency is related to the quinolyl ring and O-phenoxy conjugated o-difluorophenyl moiety. No crystallographic studies have been reported using Q-VD-OPh, in relation to the caspase enzyme, however it remains the strongest-known peptide caspase inhibitor. The following table (Table 2) summarizes the potency of peptide-based caspase inhibitors based on the DNA ladder assay of human jurkat T-cells.

Table 2 – Structure-Potency of peptide-based inhibitors

<table>
<thead>
<tr>
<th>Structure</th>
<th>Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-VAD-fmk</td>
<td>&gt;50 µM</td>
</tr>
<tr>
<td>Boc-D-fmk</td>
<td>50 µM</td>
</tr>
<tr>
<td>Boc-VD-OPh</td>
<td>10 µM</td>
</tr>
<tr>
<td>Z-VD-OPh</td>
<td>~5 µM</td>
</tr>
<tr>
<td>Q-VD-OPh</td>
<td>≤5 µM</td>
</tr>
</tbody>
</table>

* Based on DNA Ladder of Actinomycin D induced apoptosis of Human Jurkat T Lymphocytes

Studies have also shown that Q-VD-OPh can pass through the blood-brain barrier, is non-toxic at high concentrations and is stable in vivo in a mouse model. Unfortunately, peptides are not very useful as therapeutics. They have very high manufacturing cost, are structurally large with high molecular weight, are not easily modifiable and are hydrolytically unstable in vivo. Therefore, the oxindoles were designed to mimic the structural features of Q-VD-OPh and be used in a combinatorial library to be screened for apoptotic targets. The use of the oxindole scaffold as the caspase inhibitor was not a random occurrence. Oxindole was chosen because of the effectiveness of the molecular cousin of oxindole, isatin, as a caspase inhibitor.
III. A SMALL MOLECULE APPROACH TO CASPASE INHIBITORS

A few years after the initial development of the classical peptide inhibitors, GlaxoSmithKline published an approach to creating a viable small molecule caspase inhibitor. A high-throughput screen of their entire molecular library in 2001 led to the discovery that some members of the isatin family (indolin-2,3-diones) exhibit selective inhibition towards caspase-3 and -7 with some reported IC$_{50}$ values in the vicinity of 2.5 nM.$^{56}$

These inhibitors had only two points of variability in the scaffold, namely the 5-position and N-position, capable of being functionalized in order to create more active entities. The publication$^{56}$ describes a correlation of caspase inhibition with the electron-withdrawing groups (EWG) at the C-5 position with respect to sigma constants, wherein electron-donating (EDG) groups demonstrated far less inhibition capacity.$^{56}$ It was also determined that 5-nitroisatin with an N-benzylic substituent produced very effective inhibition compared to N-H analogues, and was used as the lead compound for a drug discovery program (Figure 5).

![Figure 5 - Lead GSK nitro isatin](image)

Following the discovery of the lead compound (e.g., the 5-nitroisatin), a sulfonamide group was then used to replace the nitro moiety at the 5-position. This
allowed for more combinatorial input because a sulfonamide can be easily modified and was deemed more stable than a nitro while still maintaining strong electron-withdrawing characteristics.\textsuperscript{56} The initial compounds were sulfonamides derived from cyclic amines of varying ring sizes (4-7, i.e. $n = 1-4$) and it was determined that the 4-membered ring was most effective\textsuperscript{56} and the 5-membered ring also potent (Scheme 4).

\begin{center}
\begin{tikzpicture}
\node (A) at (0,0) {\includegraphics[width=1.0\textwidth]{scheme4.png}};
\node (B) at (0,-1.5) {Scheme 4 – GSK Development of the isatin sulfonamide};
\end{tikzpicture}
\end{center}

Therefore, given the availability of both enantiomers of the amino acid proline (\textit{vide infra}) the pyrrolidine (5-membered ring) derivative was chosen as the basis for further modifications.\textsuperscript{56} Reduction of the carboxylic acid of proline at the 2’-position of the pyrrolidine ring to the methylene alcohol was conducted with both R and S isomers. Alkylation of the hydroxyl with simple alkyl and benzylic halides afforded the methylene oxy derivatives. The molecules were alkylated at the N-position in order to investigate the connection between hydrophobic and hydrophilic substituents in the active site, and their inhibition constants. (Scheme 5).

\begin{center}
\begin{tikzpicture}
\node (A) at (0,0) {\includegraphics[width=1.0\textwidth]{scheme5.png}};
\node (B) at (0,-1.5) {Scheme 5 – GSK evolution of caspase inhibitor};
\end{tikzpicture}
\end{center}

Isomers with R-stereochemistry were shown to be many factors of 10 less effective.\textsuperscript{56} The best inhibitor (Figure 6) came from a 5-position tertiary sulfonamide\textsuperscript{56} \([(S)-\text{CH}_2\text{OPh}\]
derivative] with a benzylic substitution at the N-position with IC\textsubscript{50} reaching 2.5 nanomolar.

![Figure 6 - GSK Isatin Sulfonamide](image)

In summary, the structure-activity relationship developed by GSK indicated five main structural features important to the molecule as a caspase inhibitor.\textsuperscript{56} The first is the EWG sulfonamide located at C-5 (Figure 6). Two, pyrrolidine derivatives showed good potency, three, the (S)-enantiomer of the 2'-alkylated pyrrolidine sulfonamide showed greater efficacy over the (R) stereochemistry (Figure 6). Fourth, N-benzyl showed improved efficacy over N-H variants and finally, substituents on the N-benzyl did not translate to improved inhibition potential.

As described earlier, the direct reductive product of isatin is oxindole (indolin-2-one). Oxindole (1) has many advantages over isatin (2) in terms of combinatorial potential. The reduced carbonyl affords an available set of α-hydrogens for condensation with aldehydes, or Michael acceptors and other various electrophilic entities. Additionally, the ability of oxindoles to be easily substituted at the C-3, and C-5 positions makes the oxindole a very important scaffold for drug discovery.
This project is aimed at three main goals. The first goal is to develop a small library of aryldene oxindoles with the idea that a three-point variable oxindole could be more useful as a scaffold than analogous isatin. The second goal is to employ NMR to judge the benefits of aryldene structural modifications to the design of caspase inhibitors especially as regards the ability to modulate their Michael acceptor properties. The final goal is to determine the efficacy of aryldene oxindoles as anti-apoptotic drugs.

IV. RESULTS - SYNTHESIS

The success of the GSK study and two-point variability of isatin led into the idea at WSU that the three-point variability scaffold (through modification of the N-, C-3 and C-5 positions of oxindole) could produce a more desirable caspase inhibitor (Figure 7). The WSU molecule presented in Figure 7 below is the desired target structure where Ar = aryldene ring and R = benzylic substitution, and the development of this molecule is described herein.

![Figure 7](image)

**Figure 7** – Potent GSK isatin (left) with proposed WSU oxindole (right)

The strategy for accomplishing the synthesis of molecules with this three-points of variability is: a) C-5-position functionalization of the isatin nucleus if necessary; b) N-alkylation of the isatin; c) reduction of isatin to the corresponding oxindole, and: d) aldol
condensation of the oxindole. This can be accomplished in three synthetic steps (Scheme 6).

![Scheme 6 – Synthesis of trisubstituted oxindoles](image)

**A. INCORPORATION OF EWG AT 5-POSITION**

Previous results from the Ketcha group in 2010 showed that a 5-nitro-substituted arylidene oxindole had potent biological activity against actinomycin D-induced apoptosis of human jurkat T-cells (Figure 8).

![Figure 8 – WSU 5 µM active compound](image)

However, nitroaromatics are metabolically unstable and can be reduced to toxic aromatic amines and so further development of the nitro oxindole derivatives was not pursued.

Multiple attempts at creating sulfonamide derivatives of oxindole at the 5-position were very unsuccessful, following the published procedure by GSK. Brown crystals would form from the first step of the reaction (chlorosulfonic acid and oxindole) in a very oily mixture. This mixture was filtered and the crystals were added to an ethanol/ammonium hydroxide solution and stirred overnight. White powder would form and be isolated. However, multiple attempts at characterization of the product were unsuccessful. The products typically had broad melting point ranges and were insoluble
in GC/MS solvents (acetone) and largely insoluble in the deuterated solvents (DMSO, acetone, chloroform) employed for NMR analysis. Several independent researchers in the group repeated this procedure with similar results; therefore the C-5 sulfonamide class of derivatives was not pursued.

The desire for an electron-withdrawing group at the 5-position (by analogy to the case of the analogous isatins) led to the idea that a simple halogen substitution might prove to be just as effective. Comparing the sigma-meta constants of chlorine, bromine and fluorine,\(^{38}\) (Table 3) it was shown that they have values of 0.37, 0.37 and 0.34 respectively compared to the nitro, sigma meta of 0.71.\(^{38}\) Chlorine compared to the other halogens is smaller, more stable and cheaper, therefore the chloro substituent was elected to be used at the 5-position.

**Table 3 – The comparison of sigma meta constants for EWG**

<table>
<thead>
<tr>
<th>EWG</th>
<th>Sigma</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>0.37</td>
</tr>
<tr>
<td>Cl</td>
<td>0.34</td>
</tr>
<tr>
<td>Br</td>
<td>0.34</td>
</tr>
<tr>
<td>I</td>
<td>0.35</td>
</tr>
<tr>
<td>NO(_2)</td>
<td>0.76</td>
</tr>
<tr>
<td>(R)(_2)N-SO(_2)</td>
<td>N/A*</td>
</tr>
</tbody>
</table>

*The sigma-meta constant for sulfonamide was unavailable*

**B. WOLFF-KISHNER REDUCTION**

Before the 3-position could be used for substitution, the ketone group had to be reduced into a methylene. While several reducing agents can be employed, the amide carbonyl had to remain unscathed. Therefore, the simplest chemical approach was to use the Wollf-Kishner reduction and this is shown in Scheme 7 for the desired target molecule.
Thus, the first attempt at the Wolff-Kishner reduction of isatin involved use of 30% hydrazine hydrate (10 mL) and 1.5 g of isatin (10 mmol), which were refluxed for 30 min (Scheme 8).

What was assumed to be product (yellow solid) was collected and the rest of the reaction mixture was extracted into EtOAc. Thin-layer chromatography of starting material (isatin) and desired product (oxindole) versus reduction product (yellow ppt) and organic layer had revealed multiple byproducts in the organic layer, and a presumed “product spot” from the yellow precipitate that did not match the desired oxindole. Additionally, the melting point of the yellow ppt. was 221-223°C, well above the reported melting point of oxindole. It was later revealed by simple reasoning that the hydrazone had formed and a comparison of the literature melting point range confirms this notion. What had occurred was unsuccessful nitrogen elimination and a thus new approach to the reaction was needed.

The new method that had been examined was loosely based on the published procedure in Synth. Commun. which calls for the use of 98% wt. hydrazine hydrate with no added base (such as KOH), and short refluxing times (15-30 min). Reported yields
from this method are greater than 75%. Cautious of the possible dangers of concentrated hydrazine, it was chosen to employ 80% wt. hydrazine hydrate in the second test reaction (Scheme 9).

![Scheme 9 – Second attempt at Wolff-Kishner reduction](image)

The 10 mmol of isatin was maintained, and now instead of 10 mL of 37% wt. hydrazine, 20 mL of 80% wt. hydrazine was used. Upon immediate contact with hydrazine hydrate the isatin formed a green coating on the powder. Approximately 8 minutes into the reaction yellowing of the solution and powder was occurring and at 13 minutes an extreme bubbling of the solution was noted (indicating evolution of a gas). Twenty minutes into the reaction a red solution was noted while a layer of solid yellow precipitate was observed floating at the liquid-air interface. The reaction was continued for 12 more minutes and revealed a golden-brown solution with all solid dissolved back into the reaction mixture. At 40 minutes since by inspection no changes had occurred, the reaction was understood to be complete, according to the Synth. Commun. publication which refluxed for only 30 minutes. The reaction mixture was allowed to cool to room temperature and poured onto ice and extracted into EtOAc, dried and evaporated revealing red oil. TLC and GCMS of this red oil indicated once again the presence of multiple byproducts along with unreacted starting material (Scheme 10).
Therefore, more modifications were needed to make this experiment viable.

A publication in *J. Chem. Educ.* utilized microwave technology, which allowed the use of a lower wt. percent hydrazine hydrate. The method employed called for an 850W microwave reactor running at “medium power” with isatin and 55% wt. hydrazine hydrate paired with ethylene glycol that was irradiated for 30s generating the hydrazone intermediate. The hydrazone intermediate was isolated and added to another reaction vessel containing ethylene glycol and KOH. The new reaction mixture was irradiated for a total of 20s. Standard extraction and drying workup led to a yield of 32.4% of oxindole. While reaction times are less than one minute, yields are particularly poor and the messy use of toxic compounds such as hydrazine in open vessels as well as the multi-step nature of this procedure makes this method undesirable. However, for proof of concept, microwave irradiation was attempted (Scheme 11).

In this attempt, isatin was added in a 10 mL microwave vial with 4 mL of 80% wt. hydrazine. The microwave was pulsed at 300W for 7 min at 100°C then ramped to 120°C for 5 min followed consecutively by 5 minutes at 140°C and 10 minutes at 160°C. The
reaction sequence completed to reveal hydrazone had formed on the sides of the reaction vessel and did not further react with the hydrazine.

After several such unsuccessful attempts at reduction of isatin and derivatives a new approach to this reduction was taken. The final and most successful experimental procedure for this research followed a modification of the original Wolff-Kishner and *Synth. Commun.*\(^5\) publications. The modifications are as follows: hydrazine hydrate 80% wt. was used instead of the much more dangerous 98% wt. hydrazine hydrate and no added base was introduced (Scheme 12).

![Scheme 12 – Successful reduction of isatin with improved method](image)

Reaction times were longer, 3 hours, instead of the reported 15-30 minutes. Instead of extraction and drying, the reaction mixture was acidified, filtered and the product was obtained as it precipitated in the filtrate. Moreover, it was found advantageous to wrap aluminum foil around the reaction vessel in which case the faster/higher heating temperatures of the vessel allowed for the hydrazone to dissolve back into solution and for the reaction to continue with loss of nitrogen to afford the desired oxindole. In a typical procedure, 5-chloro-indolin-2,3-dione and 80% wt. hydrazine hydrate were refluxed for 3 hours and decanted onto ice. The mixture was neutralized, filtered and the filtrate was acidified with dilute hydrochloric acid and left undisturbed overnight. In the case of 5-chloro-oxindole (64), formation of dark brown crystals was noted. The reaction is moderately effective, yielding 65%, of a product with a melting point of 193-195˚C, which agrees with literature of 193-195˚C\(^{49}\). \(^{13}\)C NMR determination indicated eight
carbons with delta values ($\delta$) in ppm that compared well to literature values$^1$: 175.97 (C2, lit. 176.3), 142.55 (C7a, lit. 142.7), 128.04 (C5, lit. 128.1), 127.17 (C6, lit. 127.4), 125.09 (C3a, lit. 125.5), 124.46 (C4, lit. 124.6), 110.29 (C7, lit. 110.5) and 35.79 (C3, lit. 36.0).

Table 4 lists the results of the Wolff-Kishner reduction study.

![Chemical structure](image)

**Table 4** – Results of the Wolff-Kishner study

<table>
<thead>
<tr>
<th>Product</th>
<th>R1</th>
<th>R2</th>
<th>Reaction Time</th>
<th>Yield %</th>
<th>Precipitation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>H</td>
<td>H</td>
<td>3h</td>
<td>86.5</td>
<td>Overnight</td>
</tr>
<tr>
<td>64</td>
<td>H</td>
<td>Cl</td>
<td>3h</td>
<td>65.75</td>
<td>Overnight</td>
</tr>
<tr>
<td>65</td>
<td></td>
<td>Cl</td>
<td>3h</td>
<td>70</td>
<td>Immediate, oily</td>
</tr>
<tr>
<td>66</td>
<td></td>
<td>Cl</td>
<td>1.5h</td>
<td>92.78</td>
<td>Immediate, oily</td>
</tr>
<tr>
<td>67</td>
<td></td>
<td>Cl</td>
<td>3h</td>
<td>&lt;10</td>
<td>None, extraction required</td>
</tr>
</tbody>
</table>

N-Alkylated oxindoles are very oily in nature when heated and require standing overnight for solidification and to become useable as starting materials. However, this oily nature actually allows for the product to precipitate out of the hydrazine solution as soon as the reaction is complete. For N-alkylated oxindoles, this eliminates the use of extraction and/or acidification to obtain pure product. This one-pot reaction is high to moderate yielding. The oxindole (63, 64) that is un/monosubstituted requires an overnight precipitation time and acidification of the reaction mixture, increasing the amount of opportunity for non-quantitative transfer that can explain why yields significantly decrease from N-alkylated products. Attempted reduction of the N-ethyl ester is
apparently accompanied by hydrolysis of the ester functionality providing a compound so soluble in water that even with copious amounts of extraction yields are less than 10% yield.

C. ALDOL CONDENSATION

Considering that the action of isatins as caspase inhibitors is believed to involve addition of the catalytic cysteine residue of the enzyme to the ketone carbonyl of the isatin, it was decided to investigate the cell-death inhibitory properties of the structurally related 3-benzylidene-indolin-2-ones (Figure 9).

![Figure 9 – Comparison of isatin cysteine attack and Michael attack for oxindole](image)

This oxindole scaffold can be thought of as representing an $\alpha,\beta$-unsaturated Michael acceptor motif, wherein attack of the “soft” cysteine sulfur atom would be expected at the $\beta$-carbon of this $\alpha,\beta$-unsaturated system. Moreover, relative to isatins, the oxindole scaffold has the added advantage of incorporating an additional site of diversity so as to facilitate modulation of properties such as selectivity amongst caspases and bioavailability. The 3-substituted indolin-2-ones may exist as either the $Z$ or $E$ isomer about the exocyclic double bond depending on the characteristics of the substituents at the C-3 position. This will be explained in the NMR section.
Products from the reaction previously described were used as starting materials for the subsequent mixed aldon condensations. The mixed aldon condensation of indolin-2-ones has been previously reported many times\textsuperscript{29,22}. The experimental procedure used throughout this work followed a 1998 \textit{J. Med. Chem.}\textsuperscript{22} publication. For the majority of this study the 5-Cl-oxindole and associated aryl-aldehyde were thoroughly mixed in ethanol at 90°C for 3 hours (Scheme 13). Catalytic amounts (0.1 molar equivalents) of piperidine were used to catalyze the generation of the enolate anion. The mixture was cooled and the precipitate was collected by vacuum filtration.

When deciding which compounds to synthesize in the aldon condensation, two factors were considered. One, it was sought to examine if the β-position could influence Michael acceptor properties and this electronic characteristic could be assessed by NMR. Two, obtain biological activity based on the ideas that were taken from the knowledge gained in the Ketcha group from a colleague\textsuperscript{37} in 2010. In the 2010 study, a variety of 5-nitro-3-arylidene oxindole compounds were synthesized to utilize the small-molecule scaffold of oxindole in concert with structural features relevant to Q-VD-OPh to produce a viable anti-apoptotic drug. For simplicity, only the aryl ring of the arylidene oxindoles in the 2010 study will be mentioned: 2-pyrrolyl, \textit{o}-chlorobenzylidene, \textit{o}-
trifluoromethylbenzylidene, \( o-2,6 \)-difluoro benzylidene and \( m-2,6 \)-difluorobenzylidene (Figure 10).

![Molecular structure](image)

**Figure 10** - Arylidene oxindoles synthesized in 2010. (\( X = \text{NO}_2 \))

These derivatives have a strong electron withdrawing-group at the 5-position, and no N-substituents. The electron-withdrawing group, as shown by GSK\(^{56}\) is remarkable in producing strong inhibitors, which is why here the nitro group\(^{37}\) was used. The \( o-2,6 \)-difluoro moiety was used because of the structural relationship to Q-VD-OPh, other halogenated benzylidene rings were used to see if the differing halogens had any specific effect on efficacy. As it turns out, the only compound with any true notable efficacy was the 5-nitro oxindole with a \( o-2,6 \)-difluoro benzylidene moiety (Figure 11) was active at 5 \( \mu \text{M} \) against actinomycin D-induced apoptosis\(^{37}\), where the \( m-3,5 \)-difluoro molecule was inactive.

![Molecular structure](image)

**Figure 11** - Active 5-nitro arylidene oxindole from 2010\(^{37}\)
Therefore, in this current study, monosubstituted aryl aldehydes with various electron-withdrawing and electron-donating groups were utilized in the final step of the synthetic sequence to produce the 5-chloro arylidene oxindoles (Figure 12).

From this set of synthesized compounds, two compounds were elected to be screened for activity against actinomycin D-induced apoptosis. The first compound was the 5-chloro-3-(2,6-difluoro-benzylidene)-indolin-2-one, 73, as it appeared directly compared to the active compound from 2010.

The results from the assay showed this compound was negative for biological activity, as all cells had died during the test (see biological results). This was unexpected because the 5-nitro compound with the 2,6-difluoro nucleus at the same position produced exceptional results. Another arylidene oxindole that incorporates a 4-pyridine arylidene at the 3-position had been proven to be marginally active in 2010\textsuperscript{37} with 50 µM
inhibition of actinomycin-D induced apoptosis. The 50 µM compound was mimicked while replacing the 5-position hydrogen with the 5-chloro EWG to give the 5-chloro variant 70.

This compound 70 was screened and shown to be also active at 50 µM. Learning from the activity of the previous result from the Ketcha laboratory, and with the understanding that when GSK alkylated the nitrogen\textsuperscript{56} of the active isatin, there was an at least 10-fold increase in activity. It was decided that the 2,6-difluoro moiety (found in Q-VD-OPh and the 5 micromolar oxindole from 2010) should be included in the drug and was then placed on the molecule at the nitrogen as a benzyl substituent. A colleague synthesized an arylidene oxindole with the 2,6-difluoro-benzylidene ring on the nitrogen along with the 4-pyridine arylidene ring at the 3-position and created N-(2,6-difluoro-benzyl)-3-(pyrid-4-yl-methylene)-indolin-2-one 70b.
This compound was screened in the same DNA ladder assay and was shown to have activity of approximately 15 µM. From the results of species 70, shown to be active at 50 µM and the N-(2,6-difluorobeznyl) compound (70b, 15 µM) the 5-chloro variant was synthesized to produce N-(2,6-difluoro-benzyl)-5-chloro-3-(pyrid-4-yl-methylene)-indolin-2-one 74, which was determined to be very potent with 90% inhibition at 10 µM when inhibiting actinomycin D-induced apoptosis of human jurkat T-cells.

Full details of these assays can be seen in the biology section of this report (Section VI).

The results of the aldol condensations performed in this study are no more remarkable than previously reported in literature. There was a notable color change with each reaction that was used as a general indication of reaction progress. In most cases, removal of a small amount of solvent under reduced pressure relative to the total concentration of the solution, precipitation of product was immediate. In some cases, precipitation of product occurred prior to reducing the volume of solvent. Products were vacuum filtered and no purification was necessary, as shown by NMR, GC/MS and elemental analysis. The following table (Table 5) lists the arylidene oxindole species

![Chemical Structure](image-url)
that were synthesized from various aryl aldehydes when condensed with 5-chloro oxindole.

Table 5 – Arylidene oxindole products

<table>
<thead>
<tr>
<th>Arylidene (Ar)</th>
<th>Base (eq.)</th>
<th>Time</th>
<th>Yield %</th>
<th>GCMS</th>
<th>mp °C</th>
<th>Isomeric**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>68</td>
<td>0.1mol</td>
<td>180 min</td>
<td>74.5</td>
<td>256</td>
<td>246</td>
</tr>
<tr>
<td>69</td>
<td>0.1mol</td>
<td>180 min</td>
<td>58.8</td>
<td>256</td>
<td>242</td>
<td>Y</td>
</tr>
<tr>
<td>70</td>
<td>0.1mol</td>
<td>180 min</td>
<td>52.4</td>
<td>256</td>
<td>240</td>
<td>Y</td>
</tr>
<tr>
<td>71</td>
<td>0.1mol</td>
<td>180 min</td>
<td>29.5</td>
<td>286</td>
<td>219</td>
<td>Y</td>
</tr>
<tr>
<td>72</td>
<td>0.1mol</td>
<td>180 min</td>
<td>62.0</td>
<td>290</td>
<td>252</td>
<td>Y</td>
</tr>
<tr>
<td>73</td>
<td>0.1mol</td>
<td>180 min</td>
<td>77.1</td>
<td>291</td>
<td>242</td>
<td>N</td>
</tr>
</tbody>
</table>

*Screened for biological activity, **based on NMR data (See NMR section)

Base (eq.) refers to the amount of piperidine used; in all cases it was 0.1 molar equivalents. Time indicates the amount of time the reaction mixture was heated at 90°C. Yield (%) refers to the overall yield of the reaction; GC/MS indicates the molecular ion present, and m.p. is the uncorrected melting point. Isomeric (Y/N) is indicative if there are isomers found in NMR. The poor yields from the methoxy substituent can be
attributed to the strong electron-donating effect into the aldehyde carbon causing an increase in electron density making it less electrophilic.

**D. SYNTHESIS OF ACTIVE COMPOUND**

Synthesis of the active compound was achieved with ease through the methods previously described. The following scheme (Scheme 14) is a representation of the synthesis involved in synthesis of the active compound 74.

![Scheme 14 – Synthesis of the active compound](image)

Wolff-Kishner reduction of N-(2,6-difluorobenzyl)-5-chloro-indolin-2,3-dione that led to the oxindole 66 was performed in 80% wt. hydrazine hydrate (15 mL) and approximately 2 grams of starting material. After 2 hours of reflux there was oily precipitate floating in the reaction mixture. The reaction mixture was cooled and the oily mixture filtered off and let dry overnight (92% yield). Subsequent GC/MS revealed pure product and the obtained N-(2,6-difluorobenzyl)-5-chloro-indolin-2-one (66) was condensed in EtOH with 1.2 equivalents of 4-pyridine carboxaldehydhe and 0.1 equivalents of piperidine to generate the enolate and refluxed for 5 hours. The reaction mixture was immediately concentrated under reduced pressure and filtered revealing 74.
as a yellow powder which was washed with cold ethanol. The compound was then 
screened in a DNA ladder assay of actinomycin D-induced apoptosis of human jurkat T-
cells with very remarkable results. The results can be found in the biology section 
(section VI).

V. NMR RESULTS – BETA CARBON AND ISOMERIZATION

A portion of this study is related to the idea that the substrates could be used as 
Michael acceptors via cysteine addition. It was hypothesized that varying substituents 
geminal to the beta carbon could cause a shift in electronic characteristics. It was also 
investigated whether or not separation of isomers was necessary for biological testing due 
to the vehicle used being DMSO, as DMSO has previously been shown to cause 
isomerization of arylidene oxindoles. DMSO is often used as the “vehicle” in biological 
testing due to its low toxicity relative to other organic solvents, and the ability to 
transport drug directly into biological media (assists in solubility). Determining whether 
or not addition of different substituted arylidene rings at the 3-position would potentially 
shift the β-carbon towards a more susceptible target of Michael additions was 
investigated with the tools of 

13C and 1H NMR. All NMR samples were prepared at 
concentrations of 10-30 mg/mL in deuterated DMSO (DMSO-d6) run at room 
temperature on a Bruker Avance 300MHz instrument, with data collected in TopSpin. 
The first determination made was the 13C chemical shifts of 5-chloro-indolin-2-one 
(Figure 13, species 64) and a comparison to known literature values to get a baseline of 
chemical shifts relative to the oxindole scaffold.
Figure 13 – $^{13}$C NMR of 64

After determining the baseline chemical shift of 5-chloro-indolin-2-one (64), a number of synthesized 5-chloro-3-arylidene oxindoles (68-74) were examined. The majority of these compounds were isomeric based on the NMR data (See Appendix NMR). It was then reasoned that a baseline chemical shift for a non-isomeric 5-chloro-3-arylidene oxindole had to be made in order to appropriately determine the chemical shift of other arylidene oxindoles differentiating between actual/isomeric carbons and determining between $E/Z$ isomers. A solution to this problem was found in (E)-5-chloro-3-(((pyridin-2-yl)benzylidene)indolin-2-one (68). As demonstrated by Figure 14 below, the 2-pyridine compound 68 is clearly one isomer and viewing the spectra within the aromatic region with the carbonyl (100-170 ppm) shows that all 14 carbons are accounted for.

Figure 14 – Compound 68 aromatic region + carbonyl
Comparing 68, the 2-pyridine-5-chloro compound, with the baseline spectrum of 64, a 5-chloro-oxindole (Figure 13) and using DEPT135 (not shown), the scaffold carbons can be differentiated from the arylidene carbons. Table 6 below displays the relative chemical shifts of the disubstituted arylidene oxindole 68 with the baseline 5-chloro oxindole 64.

Table 6 – Chemical Shift of 64 (5-Cl-oxindole) vs. 68 (2-pyrd.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>C2</th>
<th>C3</th>
<th>C3a</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
<th>C7a</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>175.9</td>
<td>35.79</td>
<td>125.0</td>
<td>124.4</td>
<td>128.0</td>
<td>127.1</td>
<td>110.2</td>
<td>142.5</td>
</tr>
<tr>
<td>68</td>
<td>168.9</td>
<td>123.0</td>
<td>125.0</td>
<td>124.5</td>
<td>128.3</td>
<td>127.5</td>
<td>110.9</td>
<td>142.3</td>
</tr>
</tbody>
</table>

By observation, the two carbons most affected by the addition of the pyridine ring are the C-2 and C-3 carbons. The carbonyl (carbon 2) has shifted ~7 ppm, and as expected from the change in hybridization, the methylene (carbon 3) replaced by an alpha-beta unsaturated Michael acceptor has shifted 87 ppm. It seems that the carbons on the oxindole aside from 2 and 3 remain well insulated from the effects of adding the arylidene ring to the molecule. Determination of the arylidene chemical shifts was done by process of finding carbons that seemed to match appropriately with expected chemical shifts. For example, compound 68 exists as one isomer as described previously. From the spectrum of 68, it can be determined which new carbon peaks emerged, compared to the core scaffold from 64. From there, the arylidene peaks can be selectively determined by comparing 68 to the chemical shifts in literature of known 2-vinylpyridine. The
following figure (Figure 15) should help guide the comparisons of 68 with that of 2-vinylpyridine.

![Figure 15 - 2-pyridine, species 68 carbon peak labels]

Carbons that are directly adjacent to the heteroatom (a,e) are found to have significant shifting from standard benzene ring-type carbon atoms due to the presence of the nitrogen. The carbon labeled “e” has been determined to have a delta value of 152.9 ppm where “a” has a chemical shift of 149.59 ppm. Carbon “e” has a chemical shift of 137.45 ppm, an expected aromatic shift because it is not directly attached to an electronegative atom. The carbon labeled “d” has a chemical shift of 129.06 (the most upfield) and “b” is found to have a chemical shift of 130.18 ppm. The discrepancy between carbons “b” and “d” even though both are 2 carbons removed from the heteroatom is due to the tertiary carbon that lies in between the nitrogen and carbon “d” changing the relative electronic characteristic of the latter. The carbon labeled “f” is the beta-unsaturated carbon of interest and has a chemical shift of 135.24 ppm. Compared to the known literature for 2-vinyl pyridine\textsuperscript{50} (sample run in CDCl\textsubscript{3}), the determination of chemical shifts is deemed accurate, with one minor ambiguity, which will be explained. Table 7 summarizes the literature results of the 2-vinylpyridine\textsuperscript{50} compared with the experimental results for the 2-pyridine species 68.
Table 7 – Summary of 68 arylidene $^{13}$C peaks.

<table>
<thead>
<tr>
<th>Compound</th>
<th>8*(f)</th>
<th>1'(e)</th>
<th>2'(N)</th>
<th>6'(d)</th>
<th>3'(a)</th>
<th>5'(c)</th>
<th>4'(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-pyrd. (68)</td>
<td>135.24</td>
<td>152.9</td>
<td>-</td>
<td>129.06</td>
<td>149.59</td>
<td>137.45</td>
<td>130.18</td>
</tr>
<tr>
<td>2-vinylpyrd.</td>
<td>136.97</td>
<td>155.69</td>
<td>-</td>
<td>121.19</td>
<td>149.44</td>
<td>136.37</td>
<td>122.39</td>
</tr>
</tbody>
</table>

*Labeled “8” as this carbon an extension of the core oxindole scaffold*

The ambiguity lies within the 6’ carbon (“d”) and the 4’ carbon (“b”). Those carbons for 2-vinylpyridine in the literature appear at 121.19 and 122.39 ppm respectively. However, as displayed in Figure 16 (below), the carbons in compound 68 do not appear at those chemical shifts. For reference, on the unlabeled line are DEPT of the 5-chloro-3-(pyrid-2-yl-benzylidene)-indolin-2-one, species 68. The carbons on line “1” are of 5-chloro-indolin-2-one, species 64 origin and the spectrum which appears on line “2” is the $^{13}$C of 5-chloro-3-(pyrid-2-yl-benzylidene)-indolin-2-one compound 68 origin. The floating vertical lines that appear between spectra “1” and “2” in Figure 16 are lining up CH carbons of the DEPT to those on the full $^{13}$C “line 2” spectrum.

![Figure 16 – DEPT (68); $^{13}$C “1” – 5-chloro oxindole (64); $^{13}$C “2” – 2-pyrd. (68)](image)

Carbons that appear in the 120-128 ppm range are located on the oxindole ring, as displayed by the close alignment of “1” with “2” and comparisons of the core oxindole scaffold. Therefore it is determined that carbons 6’ and 4’ do in fact have a chemical
shift about 8 ppm higher than the literature value of 2-vinylpyridine. Without having a crystal structure in hand, determining whether or not this molecule has adopted \(E\) or \(Z\) configuration is challenging. However, it is hypothesized that this molecule adopts an \(E\) conformation because of the intermolecular distance between the pyridine nitrogen and \(H_4\) of the oxindole ring and the \(H_{\text{vinyl}}\) proton hydrogen bonding with the carbonyl oxygen (Figure 17). The lone pair orbital from nitrogen in the pyridine ring is also proposed to be more repulsive to the lone pair on the oxygen atom forcing the molecule to isomerize \(E\).

![Figure 17](image) – Proposed N-H\(_4\) interaction and hydrogen bonding of \(H_{\text{vinyl}}\) and C=O

Now that a method for determining relative chemical shifts for the carbons has been made, carbon assignments were determined for synthesized 5-chloro-3-(arylidene)-indoline-2-ones (68-72). The first table presented (Table 7a) is the relative chemical shift for the oxindole scaffold of each compound. The compounds listed are the aryl aldehyde products since all molecules have the same oxindole scaffold from compound 64. When comparing carbons, Figure 18 shows that “core oxindole scaffold” refers to carbons 2-7a and the “arylidene” refers to the added aldehyde, carbons 8-6’.
Figure 18 – defining the regions of the disubstituted oxindole for $^{13}$C NMR analysis

Table 7a indicates the carbon atoms on the core oxindole scaffold, and the relative chemical shifts (in ppm) to one another. Table 7b indicates the carbon atoms on the arylidene relative to one another.

Table 7a – Core oxindole $^{13}$C relative ppm shifts for substituted arylidene oxindoles

<table>
<thead>
<tr>
<th>Compound</th>
<th>2</th>
<th>3</th>
<th>3a</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>7a</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Cl oxindole</td>
<td>175.97</td>
<td>35.79</td>
<td>125.09</td>
<td>124.46</td>
<td>128.04</td>
<td>127.17</td>
<td>110.29</td>
<td>142.55</td>
</tr>
<tr>
<td>2-pyrd (68)</td>
<td>168.95</td>
<td>123.05</td>
<td>125.09</td>
<td>124.58</td>
<td>125.31</td>
<td>127.56</td>
<td>110.9</td>
<td>142.31</td>
</tr>
<tr>
<td>3-pyrd (69)</td>
<td>166.76</td>
<td>126.23</td>
<td>127.74</td>
<td>123.23</td>
<td>129.65</td>
<td>130.05</td>
<td>110.9</td>
<td>139.73</td>
</tr>
<tr>
<td>4-pyrd (70)</td>
<td>167.68</td>
<td>121.87</td>
<td>124.65</td>
<td>125.12</td>
<td>129.55</td>
<td>130.39</td>
<td>111.03</td>
<td>142.14</td>
</tr>
<tr>
<td>4-OMe (71)</td>
<td>168.5</td>
<td>122.8</td>
<td>124.84</td>
<td>127.48</td>
<td>127.31</td>
<td>129.11</td>
<td>110.47</td>
<td>141.38</td>
</tr>
<tr>
<td>4-Cl (72)</td>
<td>168.09</td>
<td>122.18</td>
<td>125.02</td>
<td>121.83</td>
<td>127.33</td>
<td>129.84</td>
<td>111.56</td>
<td>141.82</td>
</tr>
</tbody>
</table>

Table 7b – Arylidene $^{13}$C relative ppm shifts for substituted arylidene oxindole

<table>
<thead>
<tr>
<th>Compound</th>
<th>8</th>
<th>1'</th>
<th>2'</th>
<th>6'</th>
<th>3'</th>
<th>5'</th>
<th>4'</th>
<th>7'</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-pyrd (68)</td>
<td>135.24</td>
<td>152.9</td>
<td></td>
<td>137.45</td>
<td>129.06</td>
<td>149.59</td>
<td>130.18</td>
<td>-</td>
</tr>
<tr>
<td>3-pyrd (69)</td>
<td>134.74</td>
<td>125.53</td>
<td>152.57</td>
<td>128.89</td>
<td></td>
<td>120.24</td>
<td>150.62</td>
<td>-</td>
</tr>
<tr>
<td>4-pyrd (70)</td>
<td>135.1</td>
<td>140.31</td>
<td>122.96</td>
<td>122.27</td>
<td>149.8</td>
<td>150.27</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>4-OMe (71)</td>
<td>137.9</td>
<td>126.79</td>
<td>134.77</td>
<td>131.52</td>
<td>114.38</td>
<td>113.81</td>
<td>160.86</td>
<td>55.4</td>
</tr>
<tr>
<td>4-Cl (72)</td>
<td>137.07</td>
<td>132.88</td>
<td>128.94</td>
<td>128.32</td>
<td>133.77</td>
<td>131.1</td>
<td>139.5</td>
<td>-</td>
</tr>
</tbody>
</table>

*7' is the methyl of the –OMe substituent

Now that plausible carbon assignments had been made, a determination of whether or not substituents on the aryl ring could affect the beta-unsaturated carbon as a
Michael acceptor by “tuning” the carbon towards a more electrophilic/electron poor double bond was possible. From Table 7b above, carbon 8 is the beta-unsaturated carbon of interest. These data are reproduced again in Table 8 with the average included.

Table 8 – beta-unsaturated $^{13}$C chemical shift presented with an average

<table>
<thead>
<tr>
<th>Compound</th>
<th>C8 (ppm)</th>
<th>Average (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-pyrd (68)</td>
<td>135.24</td>
<td></td>
</tr>
<tr>
<td>3-pyrd (69)</td>
<td>134.74</td>
<td>136.01</td>
</tr>
<tr>
<td>4-pyrd (70)</td>
<td>135.1</td>
<td></td>
</tr>
<tr>
<td>4-OMe (71)</td>
<td>137.9</td>
<td></td>
</tr>
<tr>
<td>4-Cl (72)</td>
<td>137.07</td>
<td></td>
</tr>
</tbody>
</table>

The varying substituents on the aryl ring do not seem to change the characteristics of the Michael acceptor by any significant margin. The average chemical shift is 136.01 ppm and the most downfield beta-carbon (most downfield from other arylidene species) is produced as expected from the 4-OMe species 71 and the strong electron-donating effects. The most upfield beta-carbon comes from the 3-pyridine species 69, probably due to the location of the nitrogen meta to the carbon attached to the Michael acceptor potentially interrupting resonance contribution.

The NMR analysis of these compounds was also able to reveal one more practical piece of information relevant to this project: isomerization. Commercial drugs typically are active as one isomer, therefore great effort is made to isolate isomers for biological testing. The DNA ladder assay in this investigation uses DMSO as a solvent for the stock solutions of drugs. Therefore, due to isomers being detected in the NMR of the arylidene species 69-73, isomerization was investigated by maintaining the 4-OMe species 71 in DMSO-$d_6$ over a period of one week. The NMR analysis of the one-week period shows there is active isomerization occurring in solution. Figure 19 demonstrates this process very distinctly.
Figure 19 – $^{13}$C NMR of 5-chloro-3-(4-methoxy-benzylidene)-indoline-2-one 71 in DMSO-$d_6$ isomerizing over a period of one week.

The compound when formed and isolated as a solid is significantly biased towards one isomer, “Day 1” of Figure 19. However after being exposed to DMSO over time, in some equilibrium-dependent process the isomers appear to become nearly equal in a 50:50 mixture. This process is also indicated in the $^1$H NMR of the methoxy-substituted 5-chloro oxindole (71, Figure 20). The proton peaks centered at 8.5 ppm, 7.9 ppm, 7.8 ppm, 7.3 ppm, 7.2 ppm, 7.05 ppm and 6.8 ppm all seem to have become more abundant in population with the other peaks of the separate isomers remaining “stable.” Therefore, the NMR spectra seem to be showing active isomerism over time.

Figure 20 – $^1$H NMR of 5-chloro-3-(4-methoxy-benzylidene)-indoline-2-one 71 in DMSO-$d_6$ isomerizing over a period of one week.
NMR can assist in determining which isomer is formed most abundantly as a solid, and the equilibrium that brings the other isomer to become almost 50:50 in quantity. The E/Z configuration can be determined by $^1$H NMR as the chemical shifts of both the vinylic and ortho-arylidene protons are significantly influenced by configuration: the vinylic proton is more deshielded in the E-isomer due to the influence of the carbonyl, whereas ortho-benzylidene protons are more deshielded in the Z-isomers for the same reason. Generally, in the Z isomer, the H-2’ or H-6’ protons occur in the range of 7.85-8.53 ppm compared to 7.45-7.84 ppm for the E isomers (Figure 21).

![Figure 21 – Schematic of E/Z isomerization in arylidene oxindoles](image)

Whereas it might be expected that the E-isomer would be favored due to the steric repulsion between the C-2 carbonyl group and the proton(s) at the C-2’ (6’, H_o) position of the phenyl ring in the Z-form, previous investigators have observed an equilibrium between the Z and E isomer forms in polar solvents such as methanol and dimethylsulfoxide. However, to date, studies examining the biological activities of 3-benzylidene-indolin-2-ones have typically separated and tested both E and Z isomers. So, by inspection of the NMR in Figure 20, it is concluded that the molecule is starting out “E” but trending towards equilibrium with the “Z” isomer based on the large increase in intensity of the peaks at ~8.5 and 7.9 ppm. Using the ideas just presented, a determination of proton-assignments can be made. The following figure (Figure 22,
compound 71) displays protons in assignments with the letters A-F, where the duplicate letters indicate there are isomers present.

![Proton assignments for 5-chloro-3-(4-methoxy-benzylidene)-indolin-2-one (compound 71). Only E isomer is drawn.](image)

**Figure 22.** – Proton assignments for 5-chloro-3-(4-methoxy-benzylidene)-indolin-2-one (compound 71). Only E isomer is drawn

The 2’,6’-ortho protons (relative to the beta carbon) “E” appear to be largely shifted by about 1 ppm. Presumably, this is due to previously mentioned deshielding effects of the carbonyl on the “Z” side of the benzylidene ring. The splitting of the doublets is 8.44 Hz. The vinylic proton assigned “D” appears as an expected singlet, due to having no neighboring protons with the expected chemical shift of 7.5-8.0 ppm for a hydrogen located on a double bond geminal to a benzene ring and a cis/trans amide linkage. The proton labeled “A” corresponds to H-4 which appears to have through-space coupling across the benzene ring to proton “B”, which is indicated with the very minor ~1.5 Hz splitting for both. Proton “B” appears as a doublet of doublets with 1.5 Hz through-space coupling to “A” and then a more general 7.5Hz splitting of the doublets themselves. Protons labeled “F” are the 3’,5’-ortho protons (relative to the beta carbon) doublets determined to have 8.5 Hz splitting. Proton “C” resides at C-7 as a doublet with splitting of 8.5 Hz. **Figure 23** displays the overall splitting pattern and integrations.
noticed within the para-substituted arylidene oxindoles, using the 4-Cl compound 72 as an example.

Figure 23 – Splitting patterns displayed by dichloro compound 72.

For a complete set of spectra, see the NMR appendix.

DMSO-induced isomerization has been acknowledged, and a firm determination on proton assignments has been acquired for these arylidene oxindoles (68-73). It is concluded from the NMR study that the beta-unsaturated carbon of interest remains well insulated from the effects of the substituents on the benzene ring by utilizing both $^{13}$C and $^1$H NMR. Separation of isomers by column chromatography for the initial stage of biological testing is not necessary due to clear DMSO-induced isomerization.
VI. BIOLOGICAL RESULTS – DNA LADDER ASSAY OF ARYLIDENE OXINDOLES

General Procedures for the DNA Ladder Assay

The DNA ladder assay tests for actinomycin D induced apoptosis by revealing fragmented cytosolic DNA in a 1.2% agarose gel as a “ladder.” Actinomycin D is a small peptide of *Streptomyces* origin that interrupts DNA machinery causing a cell to trigger the apoptotic sequence. If the cell successfully undergoes apoptosis and dies then laddering of DNA fragments is observed. The absence of laddering suggests apoptosis did not occur and the cell survived because of successful inhibition. Nuclear DNA is not produced in this assay; this technique prevents the nucleus from being broken open. The cell line consisting of human jurkat T lymphoma cells (type 2) was used in this assay, as it was used in other assays.\(^{35-37}\)

The cells were incubated in RPMI 1640 media and scaled up in a 1:5 dilution for every 50 mL of media, where the quantity of 50 mL 1:5 dilutions is dependent on the amount of cells needed for the assay. Once the cells had multiplied enough, as determined by a cell hemocytometer under an optical microscope, they were harvested by centrifugation (3 min x 3000 RPM) and removal of media. Cells were then diluted together in a serial fashion using 20 mL of fresh RPMI. For example one pellet of cells was diluted, then suspended in RPMI through vortexing and this suspension was subsequently added to a second pellet of cells, the whole mixture was then suspended in the same 20 mL of RPMI through vortexing and continued until all cell pellets were
collected. Following this, cells were then added in appropriate quantities to fresh RPMI media to equal a total 10 mL volume. The ideal concentration of cells for this technique is $1 \times 10^7$ cells/mL. For example, if one mL of media had 10 million cells, then that one mL of cell suspension was added to 9 mL of fresh RPMI to equal 10 mL. Drug was then added in at appropriate quantity and incubated for 30 min. After initial preincubation of 30 min, Actinomycin D (“AD”) at a concentration of 200 µg AD:200 µL MeOH was added in 10 µL portions per flask that required AD and then the cells were incubated (“treated”) for 4 hours. Following treatment with actinomycin D cells were collected via centrifugation (3 min x 3000 RPM) and removal of media and these cells were suspended in 700 µL HL buffer to lyse open the cells. Extraction with 700 µL tris-buffered phenol was carried out and this mixture was centrifuged for 3 min (at 3000 RPM) to aid in mixing and separation of organic/aqueous layers. The top layer was removed and saved, while the bottom layer was discarded. This new layer was then washed and extracted with chloroform-IAA-phenol mixture and centrifuged for 3 min at 3000 RPM and the top layer was saved. DNA from this top layer was then precipitated out using 650 µL iso-propanol and 60 µL 6M NaCl overnight at low temperature (-20°C). After overnight precipitation solvent was removed revealing DNA pellet. DNA Pellet was washed with cold isopropyl alcohol and then suspended in 20 µL 1x TE buffer. Then 3 µL 1x DNA Marker was added along with 1 µL RNase free DNase to the 20 µL 1x TE solution and this was warmed to 37°C for 15 minutes and then 23 µL of solution was added to each well of the 1.2% agarose gel with 1-2 µL of ethidium bromide and run for 90 minutes at 0.72V.
Reading the Gel

Initial gels were always run with the following channels (Figure 24, example gel): DNA Marker (M) was used to determine fragmentation patterns, Drug only (D) was used to test the ‘toxicity’ up to maximum concentration of 100 µM, Vehicle (V) was used as a control to see if the vehicle caused any toxic effects, and AD only (AD) was a control for apoptotic fragmentation. The numbered lanes indicate concentration of drug + 10 µL AD added.

![Figure 24 – Example Gel - M (DNA Marker); D – Drug Only (up to 100 µM); V – Vehicle (DMSO/MeOH); AD - 10 µM Actinomycin D; 20, 50, 100 refer to the concentrations of added drug plus (+) 10 µM of AD added to the solution](image)

A. INITIAL SCREENS

As described in the synthesis section, compounds from the 2010 study were determined to be active at 5 µM and 50 µM in the same DNA ladder assay.
The two compounds were initially screened in a DNA ladder assay were chosen from the library of synthesized compounds due to structural features relevant to that of Q-VD-OPh. In this study, structurally relevant compounds, species 70 and 73 respectively with 5-chloro substituents were synthesized and screened.

The 5-chloro-3-(2,6-difluoro-benzylidene)-indolin-2-one (73) was the first compound screened to inhibit actinomycin D-induced apoptosis of human jurkat T-cells (Figure 25). The 20 µM lane was unavailable due to operator error (spill) however the test conclusively shows that apoptosis was not inhibited by this compound at any concentration partially due to micro crystallization (observation under optical microscope) of compound 73 after incubation for 4h with AD.
This was an unexpected result as a 5-nitro-(2,6-difluoro-benzylidene)-oxindole was able to inhibit apoptosis at concentrations of 5 µM.\textsuperscript{37}

Utilizing the somewhat positive result from the 3-(pyrid-4-yl-arylidene)-indoline-2-one\textsuperscript{37} that was capable of inhibiting apoptosis at concentrations of 50 µM\textsuperscript{37} in 2010, a 5-chloro variant of the 3-(pyrid-4-yl-arylidene)-indolin-2-one molecule was screened for biological activity, species 70. The results indicate that the 5-chloro variant 70 is capable of inhibiting actinomycin D-induced apoptosis at concentrations of 50 µM (Figure 26). This molecule was expected to have activity based on previous results,\textsuperscript{37} but there was indication from the 5-nitro compound\textsuperscript{37} that an electron-withdrawing group at the 5-position would enhance the activity of the drug. It turns out that it remains just-as effective as the non-chloro variant.
Figure 26 – DNA ladder assay of 70. 50 μM inhibiton noted with no fragmentation

Following this result, an N-alkylated oxindole 70b was screened for biological activity (Figure 27). This oxindole was unsubstituted at the 5-position but incorporated the 2,6-difluoro moiety that was active with the 5 μM compound. It also utilized the 4’-pyridine nucleus at the 3-position that was shown to be active at 50 μM.

Figure 27 - Species 70b active at 15-20 μM
The addition of the 2,6-difluoro benzyl substitution at the N-position of species 70b enhanced the activity of the drug from 50 µM up to 15 µM (data not shown). The compound showed that inclusion of the 2,6-difluoro benzyl ring, as seen in Q-VD-OPh is what is partially responsible for increased activity.

**B. THE SYNERGYSTIC EFFECT**

The final compound tested (74) reintroduced the chlorine substituent at the 5-position and kept the substitutions the same at the N- and 3-positions as seen in species 70b. The results here are quite remarkable.

![Chemical Structure](image)

The compound 74, an N-(2,6-difluoro-benzyl)-5-chloro-3-(4-pyrid-yl-benzylidene)-indolin-2-one shows potent 90% inhibition at 10 µM and complete inhibition of actinomycin D induced apoptosis at 20 µM (Figure 28). It seems that the combined elements of the 2,6-difluoro benzylidene ring moiety along with the 5-chloro EWG substituents and the 4-pyridine nucleus all contribute to the impressive potency of compound 74.
Figure 28 – Compound 74 DNA Ladder. 90% inhibition of apoptosis at 10 µM.

A second DNA ladder was run with a second synthesis of the same molecule (Figure 29, compound 74) to prove a few things. One, the molecule synthesis could be repeatable. Two, the assay could be performed twice and also show repeatability. Finally, the assay was used to test lower concentrations of the drug for a rough estimate on the lowest concentration with inhibition.

Figure 29 - Species 74 50% Inhibition of apoptosis at 9 µM and complete inhibiton at 13 µM
C. SUMMARY OF INHIBITORS

The following table (Table 9) summarizes the potency of the developed oxindole apoptosis inhibitors in relation to other commercially available or published caspase inhibitors.

Table 9 – Comparative List of anti-apoptotic compounds

<table>
<thead>
<tr>
<th>Structure</th>
<th>Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-VAD-fmk</td>
<td>&gt;50 µM</td>
</tr>
<tr>
<td>Boc-D-fmk</td>
<td>50 µM</td>
</tr>
<tr>
<td>Boc-VD-OPh</td>
<td>10 µM</td>
</tr>
<tr>
<td>Z-VD-OPh</td>
<td>~5 µM</td>
</tr>
<tr>
<td>Q-VD-OPh</td>
<td>≤5 µM</td>
</tr>
<tr>
<td>GSK Isatin</td>
<td>10 µM</td>
</tr>
<tr>
<td>2010 Oxindole</td>
<td>5 µM</td>
</tr>
<tr>
<td>2011 Species 74</td>
<td>10 µM*</td>
</tr>
</tbody>
</table>

*Based on DNA Ladder assay. *90% inhibition at 10 µM

VII. CONCLUSION TO THE STUDY

Through the interpretation of biological data, it is now reported that a novel trisubstituted arylidene oxindole has potent anti-apoptotic properties. There were no mechanistic studies done to determine official therapeutic index, or the enzymes that have been targeted and related specificity. However, species 74 has potency in the DNA ladder assay equivalent to that of the GSK isatin. This compound was synthesized in 3 steps using commercially available 5-chloro isatin, compared to twice as many steps needed to synthesize the GSK active compound. A 3-point variable oxindole has been utilized and shows potent anti-apoptotic properties compared to the two-point variable GSK isatin. NMR studies have determined that separation of isomers is not necessary in the initial assays testing for biological activity. The NMR study also determined that the
substituents on the benzylidene ring do not seem to influence the capacity of these substrates as Michael acceptors. The species 74 successfully incorporates all relevant structural features found in Q-VD-OPh and modified important concepts discovered by researchers at GSK. It is novel in that it contains a unique scaffold, novel substitutions and potent biological activity. Until this thesis was written, species 74 has never been published in literature.
Future Work

The discovery of this anti-apoptotic drug is only a small advancement towards a better understanding of design and implementation for therapeutics against apoptotic diseases. My hope is that this small molecule will be screened for more biological data. It needs kinetic, toxicological and mechanistic data together with an animal model to really show whether or not it is truly capable of remarkable applications. Furthermore, discovery of this oxindole will hopefully lead to more structural diversification of this molecule to see if any greater efficacy can be gained. There may be a hidden use that these oxindoles hold but just need someone to open the door for more potential treatments.
Experimental

Chemical Analysis

Melting points were determined via the use of open capillaries with an Electrothermal melting point apparatus and are reported uncorrected. Elemental analyses were performed by Midwest Microlab, Indianapolis, IN. Elemental analysis results are within +0.4% of the theoretical values. The $^1$H and $^{13}$C NMR data were obtained on a Bruker Avance 300 MHz NMR in CDCl$_3$ solution unless otherwise indicated. The chemical shifts are reported in $\delta$ (ppm) downfield from tetramethylsilane as an internal standard; coupling constants ($J$) are in Hz. The following abbreviations are used to describe peak patterns where appropriate: s, singlet; d, doublet, dd, double doublet; t, triplet; q, quartet; dt, double triplet; m, multiplet. GC/MS measurements were performed using Hewlett-Packard 6890 Series GC with auto injection and mass fragments are reported as mass per charge, $m/z$. The GC was coupled with a mass spectrometer with a Hewlett-Packard 5973 mass selective detector/quadrupole system. Flash column (Silica Gel, Premium Rf, 200-400 mesh, Sorbent Technologies) and thin layer chromatography (TLC) were performed on silica gel with indicated solvent systems. All microwave reactions were performed in a monomode Biotage Emery’s Creator 300 Watt system and the MARS Glasschem 300 Watt system by CEM. It should be noted that all reactions were run with sample absorption set to “normal”. Multiple reactions were carried out using the FIRSTMATE system by Argonaut Technologies.
**Indolin-2-one (63)**

Isatin (3.0130g, 20.4 mmol) was transferred to a vessel containing hydrazine hydrate (25 mL, 80% wt.) and the mixture was heated under reflux for 2 h. The mixture was poured on ice and acidified to pH ~3 with conc. HCl (33 mL) after dilution with 25 mL H₂O. Extraction with EtOAc (3 x 30 mL) was performed and the combined organic extracts were dried over Na₂SO₄. Evaporation of solvent under reduced pressure revealed brown-white crystals as pure product. Vacuum-assisted filtration followed by drying afforded pure product: (2.3622 g, 86.5%), mp 124-126˚C (lit.² mp 127˚C). GC/MS in acetone revealed 133 m/z (M⁺, 100%).

**5-Chloro-indolin-2-one (64)**

5-Chloro-Isatin (2.4220g, 13.34 mmol) was transferred to a vessel containing hydrazine hydrate (25 mL, 80% wt.) and the mixture was heated under reflux for 4.5 h. with an aluminum foil blanket around the vessel. The reaction mixture was poured on ice and neutralized to a pH 7 with dil. HCl (4 M, 20 mL). An initial greenish ppt. was vacuum filtered and the ppt. discarded. The filtrate was further acidified to pH ~3 (50 mL) and let sit overnight. Brown crystal formation noted and these crystals were vac. filtered off to give pure product. Brown needles were dried and recovered (1.652 g, 65.8%):, mp 193-195˚C (lit.⁴⁹ mp 195˚C). GC/MS: 167 m/z (M⁺, 100%). ¹³C (300 MHz, DMSO-d₆): δ 175.97 (C2, lit¹. 176.3), 142.55 (C7a, lit. 142.7), 128.04 (C5, lit. 128.1), 127.17 (C6, lit. 127.4), 125.09 (C3a, lit. 125.5), 124.46 (C4, lit. 124.6), 110.29 (C7, lit. 110.5) and 35.79 (C3, lit. 36.0). ¹H (300 MHz, DMSO-d₆): δ 3.30 (1H, s), 6.81 (1H, d, J = 8.19 Hz), 7.20 (1H, d, J = 7.30 Hz), 7.25 (1H, s), 10.45 (NH, s), 2.4 (solvent), 3.6 (water).
N-Benzyl-5-chloro-indolin-2-one (65)

In hydrazine (15 mL, 80% wt.), N-Benzyl-Isatin was added (1.96 g, 6.36 mmol). The reaction mixture was refluxed for 1.5 h with an aluminum foil blanket wrapped around the vessel. An oily precipitate developed as it was floating at the top of the liquid surface. The reaction mixture was then allowed to cool to room temperature revealing an oily grey-brown product. This product was vac. filtered on wax paper and let dry overnight. A rock of product formed (70%) and had a melting point 88-89˚C. No NMR data (product insoluble).

N-(2,6-Difluorobenzyl)-5-chloro-indolin-2-one (66)

N-(2,6-difluorobenzyl)-indolin-2,3-dione (1.99 g, 6.37 mmol) was transferred to a vessel containing hydrazine hydrate (15 mL, 80% wt.) and heated under reflux for 3 h. with an aluminum foil blanket around the vessel. The reaction mixture was then allowed to cool to room temperature revealing an oily yellow product. This product was vac. filtered on wax paper and let dry overnight. A rock of product formed (1.7328g, 92.78%) and had a very broad melting point 120-125˚C. No NMR data (product insoluble). Elemental analysis (C_{21}H_{13}ClF_{2}N_{2}O): C: 61.34%, found 60.88%; H: 3.43%, found 3.42%; N: 4.77, found 4.88%.

N-Methyl(ethanoate)-5-chloro-indolin-2-one (67, attempted synthesis)

N-Methyl(ethanoate)-indolin-2,3-dione (0.535 g, 2 mmol) was transferred to a vessel containing hydrazine hydrate (15 mL, 80% wt.) and heated under reflux for 2 h. with an aluminum foil blanket around the vessel. The reaction mixture was poured onto ice and neutralized with dil. HCl (3M, 80 mL). This solution was cloudy-yellow and vac. filtered. The filtrate was further acidified with dil. HCl (3M, 50 mL) and let sit
overnight. Filtrate was then extracted into EtOAc (3x 50 mL) dried over sodium sulfate and solvents were evaporated under reduced pressure. Filtrate of product revealed very little product < 10 % and could not be characterized due to such insufficient yields.

5-Chloro-3-(pyrid-2-yl-methylene)-indolin-2-one (68)

In a vessel containing ethanol (25 mL), 5-chloro-oxindole (0.2562 g, 1.5 mmol) was added along with 1.2 eq. 2-pyridine carboxaldehyde (180 µL) and 0.1 eq. piperidine (15 µL) and the mixture was heated at 90˚C with vigorous stirring for 3 h. The reaction mixture was concentrated under reduced pressure and vac. filtered and washed with cold EtOH. The ppt. was dried revealing brown needles as pure product (0.2927g, 74.5%). mp 246 (lit. see patent51). GCMS: 289 m/z (M⁺, 100%) ¹³C NMR (300 MHz, DMSO-d₆): δ 168.9 (C₂), 123.0 (C₃), 125.0 (C₃a), 124.5 (C₄), 128.3 (C₅), 127.5 (C₆), 110.9 (C₇), 142.3 (C₇a), 135.24 (C₈), 152.9 (C₁'), 149.59 (C₃'-), 130.18 (C₄'), 137.45 (C₅'), 129.06 (C₆'). ¹H NMR (300 MHz, DMSO-d₆): δ 6.88 ppm (1H, d, J = 8.29 Hz), 7.35 (1H, dd, Jouter = 8.26 Hz, Jintra = 2.19 Hz), 7.5 (1H, m), 7.64 (1H, s), 8.0-7.8 (2.5H, m), 8.91 (1H, d, J = 4.55 Hz), 9.15 (1H, d, J = 1.97 Hz), 10.77 (NH, s), 3.6 (water), 2.4 (solvent)

5-Chloro-3-(pyrid-3-yl-methylene)-indolin-2-one (69)

In a vessel containing ethanol (25 mL), 5-chloro-oxindole (0.2529 g, 1.48 mmol) was added along with 1.2 eq. 3-pyridine carboxaldehyde (180 µL) and 0.1 eq. piperidine (15 µL) and heated at 90˚C with vigorous stirring for 3 h. The reaction mixture was concentrated under reduced pressure and vac. filtered and washed with cold EtOH. The ppt. was dried revealing brown needles as pure product (0.2366g, 58.8%): mp 242 (lit. see patent52). GCMS: 256 m/z (M⁺, 100%) ¹H NMR (300 MHz, DMSO-d₆): 6.85 ppm (1H, d, J = 8.29 Hz), 6.90 ppm (1H, d, J = 8.28 Hz), 7.28 ppm (1H, m), 7.6-7.48 (1H, m),
8.0-7.7 ppm (1H, m), 8.7-8.5 ppm (0.5H, m), 8.90 ppm (0.5H, m), 9.20 ppm (0.5H, s), 10.41 ppm (NH, s). $^{13}$C NMR (300 MHz, DMSO-$d_6$): $\delta$ 166.76, 126.23, 127.74, 123.23, 129.65, 130.05, 110.9, 139.73, 134.74, 125.53, 152.57, 128.89, 120.24, 150.62.

5-Chloro-3-(pyrid-4-yl-methylene)-indolin-2-one (70)

The reaction mixture containing ethanol (25 mL), 5-chloro-oxindole (0.1652 g, 1.0 mmol) was added in with 1.2 eq. 2-pyridine carboxaldehyde (0.1284g, 112 µL) and 0.1 eq. piperidine (15 µL) and heated at 90˚C with vigorous stirring for 3 h. The reaction mixture was concentrated under reduced pressure and vac. filtered and washed with cold EtOH. The ppt. was dried revealing brown needles as pure product. GC/MS 256 m/z ($M^+$, 100%), mp 239-241˚C (lit. see patent$^{53}$) $^1$H (300 MHz, DMSO-$d_6$) with isomers: 6.87 (1H, d, $J = 8.35$ Hz), 6.90 (1H, d, $J = 8.35$ Hz), 7.21 (1H, d, $J = 1.87$ Hz), 7.35-7.27 (2H, m), 7.63 (2H, d, $J = 8.08$ Hz), 7.77 (1H, s), 7.85 (0.5H, d, $J = 1.93$ Hz), 7.90 (0.5H, s), 8.10 (1H, d, $J = 5.91$ Hz), 8.69 (1H, d, $J = 5.69$ Hz), 8.75 (1H, d, $J = 5.57$ Hz), 10.83 (NH, s), 3.5 (water), 2.4 (solvent). $^{13}$C (300 MHz, DMSO-$d_6$): $\delta$ 167.68, 121.87, 124.65, 125.12, 129.55, 130.39, 111.03, 142.14, 135.1, 140.31, 122.96, 122.27, 149.8, 150.27.

5-Chloro-3-(4-methoxy-benzylidene)-indolin-2-one (71)

To ethanol (25 mL) 5-chloro-oxindole (0.1725g, 1.1 mmol) were added with 1.2 equivalents of 4-anisaldehyde (0.1633g, 137 µL) and 0.1 eq. piperidine (12 µL) in EtOH and stirred at 90˚C for 3h. Reaction mixture went through a light brown to orange-yellow color and concentrated under reduced pressure and vac. filtered. The yellow needles (0.0866g, 29.5%) were determined to have a metling point of 219-220˚C (lit. see patent$^{54}$) and GCMS revealed 285 m/z ($M^+$, 100%). $^1$H (300 MHz, DMSO-$d_6$): $\delta$ 3.85 (1H, s), 6.81 (0.5H, d, $J = 8.20$ Hz), 6.88 (0.5H, d, $J = 8.48$ Hz), (1H, d, $J = 8.77$ Hz), 7.11 (1H, d, 8.62
Hz), 7.19 (1H, dd, $J = 8.26$, $J = 1.85$ Hz), 7.56 (0.5H, d, 1.83 Hz), 7.67 (1H, d, $J = 7.70$ Hz), 7.72 (0.5H, s), 7.80 (0.5H, d, $J = 1.85$ Hz), 3.6 (water), 2.4 (solvent). $^{13}$C (300 MHz, DMSO-$d_6$): $\delta$ 168.5, 122.8, 124.84, 127.48, 127.31, 129.11, 110.47, 141.38, 137.9, 126.79, 134.77, 131.52, 114.38, 113.81, 160.86, 55.4 (-O-CH$_3$).

5-Chloro-3-(4-chloro-benzylidene)-indolin-2-one (72)

The 5-chloro-oxindole (0.173g, 1.0 mmol) were added to EtOH (25 mL) in a reaction vessel and added to this were 1.2 eq. p-chlorobenzaldehyde (1.2 mmol, 0.168g) with 0.1 eq. piperidine (15 µL). The reaction mixture was heated at 90°C for 3 h and concentrated under reduced pressure. Product was vacuum filtered and dried. The ppt. yielded 0.1789g (62%) of pure product. Melting point analysis was 252-254°C (lit. see patent$^{55}$) and GCMS revealed 290 m/z (M$^+$, bp). $^1$H (300 MHz, DMSO-$d_6$): $\delta$ 6.82 (0.5H, d, $J = 8.23$ Hz), 6.89 (0.5H, d, $J = 8.29$ Hz), 7.25 (0.5H, dd, $J = 8.18$ Hz, $J = 1.70$ Hz), 7.29 (0.5H, dd, $J = 8.30$ Hz, $J = 1.90$ Hz), 7.37 (0.5H, d, $J = 1.91$ Hz), 7.55 (1H, d, $J = 8.51$ Hz), 7.62 (1H, d, $J = 8.39$ Hz), 7.68 (0.5H, s), 7.73 (1H, d, $J = 8.52$ Hz), 7.84 (0.5H, d, $J = 1.96$ Hz), 7.93 (0.5H, s), 8.41 (1H, d, $J = 8.59$ Hz), 10.78 (NH, s), 2.4 (solvent), 3.6 ppm (water). $^{13}$C (300 MHz, DMSO-$d_6$): $\delta$ 168.09, 122.18, 125.02, 121.83, 127.33, 129.84, 111.56, 141.82, 137.07, 132.88, 128.94, 128.32, 133.77, 131.1, 139.5

5-Chloro-3-(2,6-difluoro-benzylidene)-indolin-2-one (73)

Using the compound 64, approximately 0.2455g (1.5 mmol) were added to ethanol (25 mL). The 2,6-difluorobenzaldehyde were added to the reaction mixture (1.2 eq., 0.2559g, 194.3 µL) with 0.1 eq. piperidine (18µL) and heated at 90°C for 3 h. Yellow-colored reaction mixture was concentrated under reduced pressure and vac. filtered revealing yellow ppt. (0.3366g, 81.33%). Yellow ppt. was added in hot ethanol to
dissolve impurities and hot filtered revealing pure product. GCMS 291 m/z (M⁺, bp), mp 242-244°C. ¹H NMR (300 MHz, DMSO-d₆): δ 10.72 (1H, s), 7.63-7.58 (1H, m), 7.32 (1H, d, J = 14.5 Hz), 7.31 (1H, d, J = 16.4), 7.25 (1H, s), 6.86 (1H, dd, J = 15.3 and 7.5 Hz); ¹³C (300 MHz, DMSO-d₆): δ 167.56, 161.31-157.89 (dd, J = 7.12 Hz), 143.13, 132.39, 131.97 (t, J = 10.41 Hz), 130.81, 123.19, 121.41, 120.75, 119.66, 112.27-111.65 (d, J = 24.54 Hz), 112.19-111.85 (t, J = 19.64 Hz), 110.2. Elemental analysis (C₁₆H₈ClF₂NO): C: 61.77%, found 61.69%; H: 2.76 %, found 2.70%; N: 4.80%, found 4.68%.

N-(2,6-difluorobenzyl)-5-chloro-3-(pyrid-4-yl-methylene)-indolin-2-one (74)

To N-(2,6-difluorobenzyl)-oxindole (0.1g, 0.34 mmol) in EtOH (5 mL) 1.2 eq. of 4-pyridine carboxaldehyde (38.4 µL) were added with 0.147 eq. piperidine (5 µL) in a vial and heated at 90°C for 6 h. The reaction mixture was immediately concentrated under reduced pressure revealing yellow ppt. and vacuum filtered. After drying, the yellow powder was determined to be pure product (0.033 g, 25.44%). Melting point 176-177°C, GCMS 293 m/z (M⁺). NMR analysis of product in DMSO 1H: 3.34 ppm (1H, s), 6.97 ppm (1H, d, J = 3.41 Hz), 7.12 ppm (3H, m), 7.24 ppm (1H, s), 7.40 ppm (indistinguishable), 7.64 ppm (1H, d, J = 5.53 Hz), 7.78 ppm (1H, s), 7.94 ppm (1H, s), 7.99 ppm (1H, s), 8.10 ppm (1H, d, J = 5.41 Hz), 8.72 ppm (1.5H, d, J = 5.23 Hz), 8.77 ppm (1.5H, d, J = 5.13 Hz), 5.04 (acetone), 2.4 (solvent), 3.6 (water). Elemental Analysis (C₂₁H₁₃ClF₂N₂O): C: 65.89%, found 65.66%; H: 3.42%, Found: 3.53%; N: 7.32%, found 7.34%.
References


51. See patent: WO 2004037247

52. See patent: CN 1883452 A 20061227

53. See patent: CN 1857201 A 20061108

54. U.S. Pat. Appl. Publ., 20090264494

55. WO 2005058309 A1 20050630

APPENDIX A. NMR

I. 5-chloro-indolin-2-one

II. 5-chloro-3-(pyrid-2-yl-methylene)-indolin-2-one
III. 5-chloro-3-(pyrid-3-yl-methylene)-indolin-2-one

CNMR

IV. 5-chloro-3-(pyrid-4-yl-methylene)-indolin-2-one

CNMR
V. 5-chloro-3-(4-methoxy-benzylidene)-indolin-2-one

[CNMR graph]

VI. 5-chloro-3-(4-chloro-benzylidene)-indolin-2-one

[CNMR graph]
VII.  N-(2,6-difluorobenzyl)-5-chloro-3-(pyrid-4-yl-methylene)-indolin-2-one

CNMR

HNMR