Biological evaluation of synthesized chemical entities.

6.1) Introduction of Anti-inflammatory activity

Inflammation is a multi-factorial process which reflects the response of the organism to various stimuli and is related to many disorders such as arthritis, asthma and psoriasis \(^{(1)}\). Inflammation process can be characterized by five phases that may or may not occur simultaneously, named pain, heat, redness, swelling and ultimately loss of function. They comprise a defensive body response to the invasion of an overseas material. Acute inflammation can cause several damages in tissues or organs. The anti-inflammatory potential of a certain molecule can be explored by various means, such as the analgesic effect using paw edema as model, the inhibition of pro-inflammatory cytokines (e.g. tumor necrosis factor (TNF-\(\alpha\)) and interleukin 6 (IL-6)),\(^{2}\) the effect on prostaglandin E2 and/or hialuronidase, nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and transient receptor A1 (TRPA1). Among them, Biginelli adducts have received great attention with respect to their potential as anti-inflammatory agents. Based on the duration of action and percentage of inflammation inhibition on Albino rats paw edema, the thio-analogues comprising propionic acid were found to be the most capable as anti-inflammatory compounds when compared to diclofenac sodium, a reference drug.\(^{3}\) The Biginelli derivative, which bears a 1,3,4-oxadiazol-2-yl moiety, controls inflammation process by inhibiting the carrageen induced rat paw edema by 75% after 3 hr of treatment, which was comparable to that exhibited by diclofenac sodium.\(^{4}\) Tale et al investigated the potential of the thio-analogue of Biginelli adduct (Fig.3.4) against pro-inflammatory cytokines in LPS-induced human monocytic leukemia cells (THP-1).\(^{5}\) Biological study revealed that these derivatives have promising anti-inflammatory activity (42–78% TNF-\(\alpha\) and 54–96% IL-6 inhibitory activity at 10 \(\mu\)M).
Inflammation is a local reaction of the vascular and supporting elements of a tissue to injury resulting in the formation of protein-rich exudates; it is a protective response of the nonspecific immune system that serves to localize, neutralize, or to destroy an injurious agent in preparation for the process of healing. The cardinal signs of inflammation are rubor (redness), color (heat), dolor (pain), tumor (swelling), and functionless (loss of function). The cause of inflammation includes physical agents, chemical agents, immunological reactions, and infection by pathogenic organism. Inflammation is divided into acute and chronic patterns. The characteristics of acute inflammation are the exudation of fluid and plasma proteins (oedema) and the emigration of leukocytes, predominantly neutrophils. Chronic inflammation is considered to be inflammation of prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously. Chronic inflammation includes some of the most common and disabling human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis, and chronic lung diseases. Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the choice treatment in various inflammatory diseases such as arthritis, rheumatisms as well as to relieve the aches and pain of everyday life. Classical NSAIDs exhibit their action by restricting the biosynthesis of prostaglandin, some of which are pro-inflammatory. This is essentially brought about by inhibiting the rate-limiting cyclooxygenase (COX) enzyme involved in the inflammatory cascade. Among different types of NSAIDs, imidazole and fused
imidazole with six-membered rings which occupy central position are used as an analgesic and anti-inflammatory agents\(^\text{10}\)

**Table 1 Anti-inflammery Activity**

Percentage inhibition of carrageenan-induced paw oedema (EJ115A-EJ115U)

<table>
<thead>
<tr>
<th>Code</th>
<th>1 hour</th>
<th>% Inhibition</th>
<th>2 hour</th>
<th>% Inhibition</th>
<th>4 hour</th>
<th>% Inhibition</th>
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<td>EJ115-A</td>
<td>6.30±1.85</td>
<td>55.60</td>
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<td>8.48±1.44</td>
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<td>EJ115-B</td>
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<td>8.81±1.22</td>
<td>42.53</td>
<td>10.47±1.33</td>
<td>43.03</td>
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<td>EJ115-C</td>
<td>10.34±1.85</td>
<td>27.13</td>
<td>11.66±1.66</td>
<td>23.93</td>
<td>13.31±1.88</td>
<td>27.58</td>
</tr>
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<td>EJ115-D</td>
<td>11.33±1.52</td>
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<td>16.82</td>
<td>14.14±1.72</td>
<td>23.06</td>
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<tr>
<td>EJ115-E</td>
<td>4.70±1.08</td>
<td>66.87</td>
<td>5.53±1.17</td>
<td>63.92</td>
<td>7.17±1.30</td>
<td>60.99</td>
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<td>EJ115-F</td>
<td>7.50±0.42</td>
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<td>9.08±0.92</td>
<td>40.76</td>
<td>10.74±1.23</td>
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<td>EJ115-H</td>
<td>8.22±0.822</td>
<td>42.07</td>
<td>9.66±0.839</td>
<td>36.98</td>
<td>10.78±0.937</td>
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<td>5.32±1.12</td>
<td>65.29</td>
<td>6.32±0.974</td>
<td>65.61</td>
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<td>EJ115-L</td>
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<tr>
<td>EJ115-V</td>
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<td>15.33±0.561</td>
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<tr>
<td>EJ115-W</td>
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<td>4.60±0.846</td>
<td>69.99</td>
<td>5.58± 0.366</td>
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## Table 2 Anti-inflammatory Activity table

Percentage inhibition of carrageenan-induced paw oedema (EJ-169-182,256-257)

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<th>Code</th>
<th>1.5 hour</th>
<th>% Inhibition</th>
<th>3 hour</th>
<th>% Inhibition</th>
<th>4.5 hour</th>
<th>% Inhibition</th>
<th>6 hour</th>
<th>% Inhibition</th>
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<tr>
<td>EJ-169</td>
<td>0.45±0.03</td>
<td>42.22</td>
<td>0.55±0.04</td>
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<td>0.64±0.02</td>
<td>46.88</td>
<td>0.75±0.01</td>
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<tr>
<td>EJ-171</td>
<td>0.26±0.04</td>
<td>37.78</td>
<td>0.30±0.01</td>
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<td>0.34±0.04</td>
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<tr>
<td>EJ-172</td>
<td>0.28±0.02</td>
<td>33.33</td>
<td>0.34±0.02</td>
<td>34.54</td>
<td>0.37±0.05</td>
<td>40.62</td>
<td>0.41±0.03</td>
<td>42.67</td>
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<tr>
<td>EJ-173</td>
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<td>28.89</td>
<td>0.36±0.04</td>
<td>30.91</td>
<td>0.38±0.02</td>
<td>35.94</td>
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<tr>
<td>EJ-174</td>
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<td>EJ-175</td>
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<td>0.37±0.06</td>
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<td>0.42±0.04</td>
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<td>EJ-257</td>
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6.2) Result and discussion of anti-inflammatory

All the 4-phenyl-2-(pyrimidin-2-yl)-1H-chromeno[4,3-d]pyrimidin-5(2H)-one derivatives (EJ-115A- EJ-115U) exhibited anti-inflammatory activity (Table 2). Compounds EJ-115J, EJ-115E, EJ-115A showed high protection against induced oedema after 360 min. whereas other compounds showed less potency. The degree of anti-inflammatory potency in ascending order was: EJ-115J, EJ-115E, and EJ-115A by % Inhibition of carrageenan-induced paw oedema (Table 2). Substitution on the 2-chloro, 4-benzailoxy benzaldehyde, 4 tert.Butyl was considered to have greater anti-inflammatory and analgesic activity. For acute toxicity, no animals died during a 48-hr. period of observation after experiments.

All the 4-phenyl-2-(pyrimidin-2-yl)-1H-chromeno [4,3-d]pyrimidin-5(2H)-one derivatives (EJ-169- EJ-257) exhibited anti-inflammatory activity (Table 1). Compounds EJ-181, EJ-180, & EJ-182 showed high protection against induced oedema after 360 min. Whereas other compounds showed less potency. The degree of anti-inflammatory potency in ascending order was: EJ-181, EJ-180 and EJ-182 by % Inhibition of carrageenan-induced paw oedema (Table 2). Substitution on the 4-floro, 2-nitro, 4-qumine,ring was considered to have greater anti-inflammatory and analgesic activity. For acute toxicity, no animals died during a 48-hr. period of observation after experiments.
### Table 3: Anti-tubercular activity

<table>
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<th>Sampal Id</th>
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<th>SD</th>
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6.3) In vitro Characterization of Anti-Mycobacterial Activity

1EMIC Under Aerobic Conditions

Introduction

The antimicrobial activity of compounds against *Mycobacterium tuberculosis* H37Rv grown under aerobic conditions is assessed by determining the minimum inhibitory concentration (MIC) of compound i.e. the concentration required to prevent growth. The assay is based on measurement of growth in liquid medium of a fluorescent reporter strain of H37Rv where the readout is either optical density (OD) or fluorescence. The use of two readouts minimizes problems caused by compound precipitation or autofluorescence. A linear relationship between OD and fluorescence readout has been established justifying the use of fluorescence as a measure of bacterial growth. MICs generated from the OD are reported in summary data. The strain has been fully characterized and is equivalent to the parental strain in microbiological phenotypes and virulence.
Chapter 6

Materials and Methods

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<th>Lot Number</th>
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<td>Supplement</td>
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<td></td>
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<td>R3501</td>
<td>SLBD2314V</td>
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<td>Recipe</td>
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<td>4.7 g/L 7H9 base broth, 0.05% w/v Tween 80, 10% v/v OADC supplement</td>
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6.3.1) Protocol

The MIC of compound was determined by measuring bacterial growth after 5d in the presence of test compounds. Compounds were prepared as 10-point two-fold serial dilutions in DMSO and diluted into 7H9-Tw-OADC medium in 96-well plates with a final DMSO concentration of 2%. The highest concentration of compound was 200 µM where compounds were soluble in DMSO at 10 mM. For compounds with limited solubility, the highest concentration was 50X less than the stock concentration e.g. 100 µM for 5 mM DMSO stock, 20 µM for 1 mM DMSO stock. For potent compounds, assays were repeated at lower starting concentrations. Each plate included assay controls for background (medium/DMSO only, no bacterial cells), zero growth (100 µM rifampicin) and maximum growth (DMSO only), as well as a rifampicin dose response curve. Plates were inoculated with *M. tuberculosis* and incubated for 5 days: growth was measured by OD$_{590}$ and fluorescence(Ex 560/Em 590)using a BioTek™ SynergyH4 plate reader. Growth was calculated separately for OD$_{590}$ and RFU. To calculate the MIC, the dose response curve was plotted as % growth and fitted to the Gompertz model using GraphPad Prism 6. The MIC was defined as the minimum concentration at which growth was completely inhibited and was calculated from the inflection point of the fitted curve to the lower asymptote (Figure 1A). In addition dose response curves were generated using the Levenberg-Marquardt algorithm and the concentrations that resulted in 50% and 90% inhibition of growth were determined (IC$_{50}$ and IC$_{90}$ respectively) (Figure 1B). Raw data is provided and can be used to plot either type of
MIC values were reported when the following quality control criteria were satisfied:

For each plate

- No growth in the background (un-inoculated) control wells
- OD\textsubscript{590}>0.2 in maximum growth wells
- Rifampicin MIC within 3-fold of the expected value

For each compound curve, MIC values were reported if

- There were 2 points with growth >75%
- There were 2 points with growth <75%

If no point reached 75% inhibition, the MIC was reported as > maximum concentration tested

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<tr>
<th>Control Compound ID</th>
<th>Strain ID</th>
<th>MIC (µM)</th>
<th>IC\textsubscript{50} (µM)*</th>
<th>IC\textsubscript{90} (µM)*</th>
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### 6.3.2) 1F MIC Against Other Disease-relevant Mycobacteria

**Introduction**

The activity of compounds against *Mycobacterium abscessus* and *Mycobacterium avium* is assessed under aerobic conditions by determining the minimum inhibitory concentration of compound (MIC). The strains are *M. abscessus* subsp. *bollettii* 103 and *M. avium* subsp. *avium* 2285 (S). The assay is based on measurement of growth in liquid medium of each strain where the readout is optical density (OD) or metabolic activity (using Alamar blue).

**Materials and Methods**

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<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Catalog Number</th>
<th>Lot Number</th>
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<td>VWR</td>
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<td>5173939</td>
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<td>Middlebrook OADC supplement</td>
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<td>Recipe</td>
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<tr>
<td>7H9-Tw-OADC</td>
<td>4.7 g/L 7H9 base broth, 0.05% w/v Tween 80, 10% v/v OADC supplement</td>
<td></td>
<td></td>
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</table>
Protocol

The MIC of compound was determined by measuring bacterial growth in the presence of test compounds. Compounds were prepared as 20-point two-fold serial dilutions in DMSO and diluted into 7H9-Tw-OADC medium in 96-well plates with a final DMSO concentration of 2%. The highest concentration of compound was 200 µM where compounds were soluble in DMSO at 10 mM. For compounds with limited solubility, the highest concentration was 50X less than the stock concentration e.g. 100 µM for 5 mM DMSO stock, 20 µM for 1 mM DMSO stock. Each plate included assay controls for background (medium/DMSO only, no bacterial cells), 100 µM rifampicin, and maximum growth (DMSO only), as well as a rifampicin dose response curve.

Mycobacterium abscessus

Plates were inoculated with *M. abscessus* and incubated for 3 days at 37°C; growth was measured by OD$_{590}$. To dose response curve was plotted as % growth and fitted to the Gompertz model. The MIC was defined as the minimum concentration at which growth was completely inhibited and was calculated from the inflection point of the fitted curve to the lower asymptote. In addition dose response curves were generated using the Levenberg-Marquardt algorithm and the concentrations that resulted in 50% and 90% inhibition of growth were determined (IC$_{50}$ and IC$_{90}$ respectively). Raw data is provided and can be used to plot either type of curve. Rifampicin was included once in each run.

MIC values were reported when the following quality control criteria were satisfied:

For each plate

No growth in the background (un-inoculated) control wells

OD$_{590}$$>$0.2 in maximum growth wells

For each compound curve. MIC values were reported if

There were 2 points with growth $>$75%

There were 2 points with growth $<$75%

If no point reached 75% inhibition, the MIC was reported as $>$ maximum concentration tested
Mycobacterium avium

Plates were inoculated with *M. avium*, incubated for 5 days at 37°C and Alamar blue was added to each well (10µL of Alamar blue to 100µL culture) and incubated for 24 h at 37°C. Plates were visually inspected and the color recorded for each well. MIC was defined as the lowest concentration at which no metabolic activity was seen (blue well). A dual read-out assay to evaluate the potency of compounds active against Mycobacterium tuberculosis.\textsuperscript{11}

Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values.\textsuperscript{12} Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplateAlamar Blue Assay.\textsuperscript{13}

MIC values were reported when the following quality control criteria were satisfied: For each plate Background (un-inoculated) control wells remain blue Maximum growth wells are pink Inhibition control wells are blue For each compound. MIC values were reported if There was a transition from pink to blue If all wells were pink, the MIC was reported as > maximum concentration tested

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<tr>
<th>Submitter Compound ID</th>
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Chapter 6

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6.4) Result and Discussion of antitubercular:

We have sent the newly synthesised compounds of chapter 5,( 5-amino-6-phenyl-[2,2'-bipyrimidine]-4-carbonitrile derivatives) for the Mycobacterium avium activity (NIH- USA) from were selected for the antitubercular study. As compared to the standard Rifampicin (which showed MIC at 5.6µM) all compounds showed moderated activity between 20-200µM Units.

6.5) Anti-cancer activity (EJ-308, EJ-319, EJ328)

Introduction: Cancer

Cancer is a leading cause of death group worldwide and accounted for 7.4 million deaths (around 13% of all deaths) in 2004. The main types of cancer are: Lung (1.3 million deaths/year), Stomach (803,000 deaths), Colorectal (639,000 deaths), Liver (610,000 deaths), Breast (519,000 deaths). More than 70% of all cancer deaths occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 11.5 million deaths in 2030 (WHO Cancer Fact Sheet, 2016). There is increasing recognition of the importance of value in the oncology drug market. The major oncology societies, American Society of Clinical Oncology (ASCO) and European Society of Medical Oncology (ESMO), have released position statements and papers outlining approaches to quantifying clinical benefit and value. Drugs approved for solid tumor treatment between 2000 and 2015 were identified and analyzed in subgroups: agents targeting oncogenes (group 1), anti-angiogenics (group 2), immunotherapy (group 3), and chemotherapy (group 4). Hazard ratios (HRs) were extracted from the registration trials and pooled in a meta-analysis.

The design of cancer chemotherapy has become increasingly sophisticated, yet there is no cancer treatment that is 100% effective against disseminated cancer. Resistance to treatment with anticancer drugs results from a variety of factors including individual variations in patients and somatic cell genetic differences in tumors, even those from the same tissue of origin. Frequently resistance is intrinsic to the cancer, but as therapy becomes more and more effective, acquired resistance has also become common. The most common reason for acquisition of resistance to a broad range of anticancer drugs is expression of one or more energy-dependent transporters that detect and eject anticancer drugs from cells, but other mechanisms of resistance including insensitivity to drug-induced apoptosis and induction of drug-detoxifying mechanisms probably play an important role in acquired anticancer drug resistance. Studies on mechanisms of cancer drug resistance have yielded important information about how to circumvent this resistance to
improve cancer chemotherapy and have implications for pharmacokinetics of many commonly used drugs $^{17}$.

Resistance to chemotherapy and molecularly targeted therapies is a major problem facing current cancer research. The mechanisms of resistance to 'classical' cytotoxic chemotherapeutics and to therapies that are designed to be selective for specific molecular targets share many features, such as alterations in the drug target, activation of prosurvival pathways and ineffective induction of cell death.$^{18}$
# Anti-cancer activity (EJ-308)

**Developmental Therapeutics Program**

**One Dose Mean Graph**

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<th>Mean Growth Percent - Growth Percent</th>
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**Report Date: Mar 22, 2017**

**Experiment ID: 17020558**

**NSC: D-795761 / 1**

**Conc: 1.00E-5 Molar**

**Test Date: Feb 13, 2017**
# Anti-cancer activity (EJ-319)

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| Mean            | 95.34          |                                     |
| Delta           | 49.96          |                                     |
| Range           | 73.67          |                                     |

**Developmental Therapeutics Program**

**One Dose Mean Graph**

**NSC:** D-795763 / 1  **Conc.:** 1.00E-6 Molar  **Test Date:** Feb 13, 2017

**Experiment ID:** 17020S58  **Report Date:** Mar 22, 2017
6.6) Result and Discussion of Anti-cancer activity:

We have sent the newly synthesised compounds of chapter 5, (5-amino-6-phenyl-[2,2'-bipyrimidine]-4-carbonitrile derivatives) for the anti cancer activity (NIH- USA) from which EJ-308, EJ-319, EJ-328 were selected for the further study. Results show moderate to low anticancer activity. Among three compounds, EJ-319 showed moderate cidal effect on Renal cancer cell line UO-31 while EJ-328 showed good cidal effect on Non-small cell lung cancer cell line EKVX. Thus, these two (EJ-319 and EJ-328) compounds may have a probable delegation for other anti cancer activity.
6.7) Reference


