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Antiproliferative Activity of Selected Non-Steroidal Anti-Inflammatory Agents: Role of Iron Complexes

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ABSTRACT

In this work the complexation of five NSAIDs with iron (Fe^{3+}) was studied and the role of these iron complexes in reducing the proliferation of cancer cells was investigated. The stoichiometry and the formation constants of the complexes formed with different NSAIDs were calculated using the conductivity method. The metal-drug ratio for all drugs was 1:2 and their formation constant values were between 10^9 to 10^{14}. The antiproliferative activity of the NSAIDs in their free and complex form was assessed using MCF-7 cells. After 72 hours incubation with the free drugs, mefenamic acid and diclofenac sodium showed the strongest antiproliferative effects with IC_{50} of 70.54 ± 15.29 μM and 108.38 ± 11.28 μM, respectively. Indomethacin, naproxen and meloxicam had moderate to no effect at the concentrations tested. A linear correlation, with r^2= 0.876, between the formation constants of NSAIDs-Fe^{3+} complexes and their cytotoxic effects was observed after 6 hours incubation. The ability of each drug to bind to DNA was examined together with the influence of ferric ions on the binding process. Drug-iron complexes were shown to bind to DNA, though with slightly different ratio. The results suggest that the complexes possess intrinsic cytotoxic effect.

Keywords: Antiproliferative Activity; Chelation; Formation Constant; MCF-7 Cells; DNA; NSAIDs.

INTRODUCTION

Non steroidal anti-inflammatory drugs (NSAIDs) are known to have chemopreventive and cytotoxic effects in addition to their anti-inflammatory action [(1-10)]. Although their anti-inflammatory action is generally believed to be a result of their cyclooxygenase inhibitory effect, the mechanism of their cytotoxic effect remains a non resolved issue [(11)]. Few suggestions have been proposed to explain the mechanism of their cytotoxic and chemo-preventive effects (with particular emphasis on colon cancer). These included apoptosis through inhibiting prostaglandin H synthase (PHS) [(9)], inhibiting cyclooxygenase (COX) activity and interfering with glucose metabolism [(12)], decreasing epidermal growth factor (EGF) in human colonic tumor [(13)], up regulating PUMA (p53 up regulated modulator of apoptosis) [(14)], up regulating expression of the tumor suppressor gene, PTEN and the MAP kinase phosphatase-3 (MKP-3) [(15)] and involvement of intracellular calcium [(16)]. Several studies have shown the ability of NSAIDs to form complexes with divalent metal ions including copper, iron and zinc [(17-22)]. In all of the reported metal complexes of NSAIDs, the carboxyl group, was shown to be involved in complex formation [(18,22,23)]. Since some metals are known to play a vital role in cell growth and development, compounds that form chelate complexes with such metals could be anticipated to interfere with some potentially essential biological processes within the cells leading to suppression

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of growth or even cell death. The ability to form chelate complexes with biologically relevant metals (particularly iron) have been implicated in the mechanism of action of some cytotoxic compounds, such as the typical iron chelator desferrioxamine [24,25] and others [26,27]. However, to the best of our knowledge, no literature reports have assessed the possibility that NSAIDs might exert their cytotoxic activity through (at least in part) formation of chelate complexes with some essential metals.

In this study we hypothesized that some of the cytotoxic activity of NSAIDs could be mediated through their ability to form chelate complexes with essential metals, specifically iron. This hypothesis was particularly attractive because, if proven, it might enhance the selectivity of such compounds towards cancer cells rather than normal cells as the former were shown to have generally higher levels of some metal ions [28]. Iron in particular has been shown to be highly required by the rapidly proliferating tumor cells as compared to their normal counterparts [29]. Studies have shown that iron, zinc and copper ions were present at higher levels in breast cancer cells than usually found in normal ones [30]. If cancer cells require essentially higher levels of relevant metals than normal, then forming complexes with such metals in the cancerous cells is expected to have more serious cytotoxic effect compared to normal ones. In this study we report the formation constants of different NSAIDs with iron and the cytotoxic effects of the potential complexes as compared to free drugs.

MATERIALS AND METHODS

Materials

Diclofenac sodium, mefenamic acid, meloxicam, indomethacin, and naproxen were donated from Hikma Pharmaceuticals, Amman, Jordan. All drugs were of purity > 99%. Ferric chloride was obtained from S.d. fine-Chem Ltd. Boisar 40/501. Calf thymus DNA (CT-DNA) and Tris buffer were obtained from Sigma (St Lois, USA). All chemicals were of analytical grade.

Equipment

Conductivity measurements were carried out using InoLab condLevel1 conductometer (TetraCon®325 , WTW, Germany) having a cell constant of 0.474cm^{-1} at 25 ºC and reported accuracy of ± 0.5%. The equipment was calibrated weekly with KCl standard calibration solution. During the course of measurement the conductivity of double distilled water did not exceed 2.2 µS/cm.

Assessment of Complex Formation

For complex formation, $3.8x10^{-4}$ M working solution of each of the NSAIDs was prepared in double distilled water (DDW). In case of mefenamic acid, indomethacin and meloxicam, suitable weights of each drug were dissolved in equimolar amounts of NaOH (3.8 mL of 0.01 M) before the volumes were completed properly to obtain a concentration of $3.8x10^{-4}$ M. Ferric chloride stock solution was prepared in DDW (pH 6.48) at a concentration of $3.8x10^{-4}$ M.

A series of solutions that contained fixed amount of the drug but increasing amounts of iron were obtained with molar ratios (r) of metal:drug in the range of 0-4. The obtained mixtures were mixed using a vortex mixer, left for 30 min at room temperature before their conductivities were measured. Electrical conductivity of each solution was measured at temperature of 37 ºC ± 0.1 and plotted against metal:drug molar ratio (r).

Calculation of Binding Constant of the Drug–Metal Complex

The overall apparent formation constants (K_f) for the formed iron complexes were calculated based on the following complexation reaction:

$$2D + M = D_2M \qquad \text{Eq.1}$$

Where D is the free non steroidal anti inflammatory drug, M is the free iron and D_2M is the formed complex (the stoichiometry of 2:1 drug:metal complex was evident from the molar ratio plot obtained experimentally in the previous section). Accordingly, K_f can be given by equation (2):

$$K_f = [D_2M]/ [D]^2[M] \qquad \text{Eq.2}$$

At any added concentration of iron to the drug, the remaining free drug [D] and free metal [M] could be determined if the concentration of the complex [D_2M] could be experimentally measured. This was enabled through conductivity measurements. The measured conductivity at

any concentration point is equal to the total conductivity attributed to different ions in solution. Since the conductivity of each ion depends on its specific conductivity and concentration, the measured conductivity can be given by the sum of the products of specific conductivity of each ion by its concentration [31]. Therefore the measured conductivity at each added concentration of iron to drug would be given by:

Total conductance = $C_{sp\ drug}[D] + C_{sp\ metal}[M] + C_{sp\ complex}[D_2M]$ Eq.3

Where the subscript (sp) indicates specific conductivity of the relevant species (drug, metal or the complex). The specific conductivity of the free drug or the metal was determined at the slope of the plot of conductance against the concentration of standard solutions of the free drug or the metal (blank) respectively. For the complex, the specific conductivity was determined from the measured conductivity value at metal to drug ratio (r) = 4 because at this ratio it could be assumed that all of the drug would be in the complex form. In order to resolve equation (3) which contains three unknowns ([D], [M] an $[D_2M]$) at any concentration point, the following two equations were established (when calculations are made at the experimentally determined stoichiometry r = 0.5):

2[D] = [M] and [D] = the concentration of added drug – $2[D_2M]$

Using the above three Equations, the three variables [D], [M] an $[D_2M]$ could be determined at r = 0.5 (the stoichiometric ratio), and thus K_f could be calculated for each NSAID when complexed to Fe^{3+}.

It is noteworthy to mention that these calculations assume negligible formation of ferric hydroxide species as a result of hydrolysis. This assumption is based on firstly, the observation that blank solutions (containing increasing amounts of ferric chloride only) did not show similar change in conductivity as was with the presence of the drug, which may indicate that $Fe(OH)_3$ did not form. And secondly, formation of $Fe(OH)_3$ (if occurred) would be expected to have a measurable effect on the conductivity values due to its very low solubility, $1x10^{-39}$ M [32].

Assessment of Antiproliferative Activity

The cell line under investigation was human breast adenocarcinoma (MCF-7 ATCC Nr: HTB 22). MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% heated foetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/mL penicillin and 50 µg/mL streptomycin. Cells were seeded with a density of 5000 cell/well in 96 wellplates and incubated for 24 h at 37 ºC and 5% CO_2. The corresponding concentrations of NSAIDs were added and incubated for 72 h for the initial determination of their antiproliferative activity. At the end of the exposure time, cell growth was analyzed using the sulphorhodamine B (SRB) assay as reported by Al-Kalaldeh *et al* [33]. Cell survival was calculated as percentages compared to controls of untreated cells. As positive controls Cisplatin and Doxorubicin (both Ebewe Pharma GmbH Nfg. KG, Austria) were used.

For investigations that involve the addition of free iron, the cells were seeded as mentioned above, after 24 h of incubation, 500 µM of ferric citrate was added for 2 h. After that the selected NSAID was added (or control) and different incubation times followed (6, 24, 48 and 72 h).

DNA Binding Studies

Solutions were prepared for each of the NSAIDs to be examined in 10 mM Tris buffer at molar concentrations of 57, 158, 172, 82 and 56 □M for meloxicam, diclofenac, naproxen, mefenamic acid and indomethacin respectively. Solutions of DNA that were employed for titration were prepared by dissolving 50 mg of the solid substance in 50 ml of 10 mM Tris buffer (containing 10 mM NaCl). The concentration of DNA was determined (as base pairs) by measuring the absorbance at 258 nm and taking molar absorptivity of CT-DNA as 7000 $M^{-1}cm^{-1}$. 900 □L of each drug solution to be tested, were placed in a micro-volume quartz UV cell and the absorbance was recorded against a proper blank. Increments of CT-DNA (50 mL each) were added to the drug solution, the solution thoroughly mixed with a micropipette after each addition, and spectra recorded (200-400 nm) after 5 minutes. Exactly similar experiments were performed, but in the presence of half molar equivalent of ferric ions, together with the drug directly before the start of titration i.e the ratio of drug to iron was kept at 2.

RESULTS AND DISCUSSION

Estimation of Formation Constants

Previous studies have shown that different diclofenac metal complexes were of low water solubility with minor shift in their UV spectra, which did not permit their study using UV spectroscopic techniques [34]. Therefore, the potential complex formation between NSAIDs and ferric ion (Fe^{3+}) was studied using conductimetric titrations. Molar ratio plots were obtained by plotting the measured conductivity of the various solutions containing a fixed amount of the drug and increasing amounts of iron against the molar ratio of added metal to drug (r). For all tested NSAIDs, the conductivity molar ratio plots exhibited inflexion points (change in the slope of the curve) at about r = 0.5, suggesting a stoichiometry of two drug molecules for one ferric ion (Fig. 1).

The observed inflexion points in the curves are a result of differences between the conductivity of free ferric ion and that of the complex. At low metal concentration, when r value is lower than 0.5, most of the drug is expected to be in the free form which has relatively lower conductivity than ferric ion because of its partial dissociation and larger size. While at higher metal concentrations, all of the drug or at least most of it is expected to be in the complex form.

The presence of inflexion points in the molar ratio plots allowed the calculation of apparent formation constants of the potential complexes formed as outlined in the experimental section [31]. A summary of the obtained stoichiometric ratios and the formation constants of the complexes is presented in Table (1).

Antiproliferative Activity of NSAIDs

The antiproliferative activity profile of the five NSAIDs under investigation was assessed by estimating the percentage of cell survival after 72 h of incubation, and dose response curves were plotted (Fig. 2). Maximal dosing was limited to 200 µM due to first, solubility limitations and second, plasma levels higher than this (up to 500 µM) are likely to have no clinical applications.

All NSAIDs appeared to have some cytotoxic activity, although significant differences in their effects could be observed; where mefenamic acid and diclofenac sodium showed the strongest antiproliferative effects with IC_{50} of 70.54 ± 15.29 µM and 108.38 ± 11.28 µM respectively. Indomethacin had moderate activity whereas naproxen and meloxicam showed no effect on proliferation in the concentration range tested.

In a second step, the antiproliferative activity was assessed at earlier incubation times (6, 24, and 48 h) and a correlation between percent living cells and formation constant with ferric ion was sought. From the time dependency plots, it was concluded that most of the differences in the antiproliferative activity of the tested NSAIDs was at the earliest time point (6 h) and this time point was further investigated to see if there was any difference in cell proliferation in the presence and absence of ferric ion added extracellularly.

After six hours of incubation, when the percentage living cells was plotted against the estimated K_f values of their iron complexes, a reasonable linearity with a correlation coefficient of (0.876) was observed (Fig. 3). This observation strongly suggests the involvement of metal (in this case Fe^{3+}) in the mechanism of the cytotoxic effect of NSAIDs.

Figure (4) compares the cytotoxic effects of NSAIDs in presence of iron to their effects in absence of iron at 6 h of incubation. Dicolfenac sodium showed enhanced antiproliferative activity in the presence of iron added extracellularly where it could be postulated that the presence of excess iron, led to the formation of extracellular complexes and those showed better permeability and enhanced cellular delivery for their antiproliferative effects. Indomethacin showed slight enhancement in its antiproliferative activity in the presence of extracellular iron, nevertheless, it was not significant. The observation that the cytotoxic activity of mefenamic acid was not potentiated in the presence of extracellular iron could be explained due to the drug's high formation constant (thousand times stronger than indomethacin or diclofenac), which means that even at quite low concentrations of metals, the drug could still be present in the complexed form. This explanation is supported by the observation that mefenamic acid exhibited highest biological activity even without ferric ion being added to the culture medium.

It is noteworthy that naproxen with its lowest estimated K_f (in the examined compounds) and meloxicam (intermediate K_f value) exhibited the lowest activity in presence and absence of extracellular iron with no detectable enhanced effect after the addition of iron.

Likely explanations for the observed effect of iron on the cytotoxic activity of the examined NSAIDs are firstly, iron forms a complex with the drug which appears to be more easily transported through cell membrane than the free drug leading to more cytotoxic effect. This suggestion is supported by the high formation constants (as estimated above) which ensures the presence of the complex rather than the free drug, and the fact that the complexed drug is significantly more lipophilic than the free form [34]. It could be concluded that the apparent cytotoxicity of the complex is similar to or even higher than that of the free drug. That accords with some previous reports that showed the increased cytotoxic effect of metal complexes of NSAIDs as compared to their free forms [17,35] and also with other reports that showed synergistic cytotoxic effect for metal ions in presence of some chelating agents [36].

Secondly, as metal complexes represent a completely different chemical entity than the free drug, then they might fit a biological target more efficiently or they may target different important biomolecules (enzymes or even DNA) that are essential for cell survival. Regardless of the exact biomolecular target, one definite conclusion is that the mechanism of action of some of the NSAIDs under investigation involves complex formation with metal ions, and these drug-metal complexes, by themselves, are involved in the cytotoxic activity. This is in contrast to the commonly advocated hypothesis for the mechanism of action of typical chelating cytotoxic agents, where the complexation process is thought to simply result in depletion of essential metal ions [37,38]. The findings of this study indicate that the iron complexes of NSAIDs are by themselves cytotoxic or at least they enhance the permeability of NSAIDs into the cells.

Further attempts were made to investigate the potential presence of a correlation between the estimated K_f values and other NSAIDs pharmacological activities that were reported in literature. A limited number of publications were found reporting quantitatively the effect (or side effect) of the selected group of NSAIDs. In one study [39] the hepatotoxic effect of some NSAIDs (namely, mefenamic acid, diclofenac, naproxen and indomethacin) was reported as percentage inhibition (% I). A plot of the reported % I against the estimated log K_f showed a reasonable correlation with a coefficient of 0.572. Although the obtained correlation coefficient was not high enough to precisely predict the hepatotoxic activity of the NSAID from K_f, it still suggests the dependence of such hepatotoxic effect on their chelation ability.

A stronger evidence on the dependence of the gastrointestinal damaging effect of NSAIDs on their metal chelation abilities could be obtained based on data from a previous study [40]. Laudanno *et al*. studied the potential of some NSAIDs to cause lesions within the small intestines. When the reported size of erosions, measured in mm^2, in the intestines was plotted against the estimated log K_f, a strikingly high correlation coefficient (0.984) was obtained. Figure (5) strongly supports the dependence of the gastro-ulcerogenic action of NSAIDs on their metal chelation ability. Although it is widely accepted that the GI side effects of NSAIDs are caused by inhibition of prostaglandins; some studies advocate the involvement of apoptotic mechanism [12,41]. Thus GIT side effects according to some authors are viewed as extension to the cytotoxic effect of NSAIDs. Consequently, the dependence of the cytotoxic effect of NSAIDs on their metal chelation ability is further supported.

DNA Binding Studies

The obtained overlaid UV spectra for the titration of each drug with DNA showed clear progressive bathochromic shifts in λ_{max} of the spectra of the drugs concomitant with a hypochromic shift (Fig. 6), which indicated binding of drug to DNA. These results, accord with previous reports which have demonstrated the potential of all tested NSAIDs to bind to DNA [42-46]. However, no previous reports have studied the influence of ferric ions complexed to NSAIDs on their binding to DNA.

Binding curves could be obtained by plotting absorbance values against molar ratio of drug to DNA (r). It was more

convenient (for comparison) to plot $\Delta A/\Delta A_{max}$ against molar ratio (where ΔA is the change in absorbance between that at a particular point and that at zero addition, while ΔA_{max} is the maximum change in absorbance obtained). Figure 7 shows the obtained binding curves for the five compounds tested. In spite of the clear differences in the binding curves of the tested compounds (in absence of iron), they all (with the exception of meloxicam) showed two breaking points, indicating two potential stoichiometry of binding (number of DNA bases to bind to one drug molecule). The stoichiometry of binding could be obtained from the binding curves as the molar ratio (r) at the intersection point of the two extrapolated lines tangenting the straight line segments of the binding curve around the breaking point [47]. With the exception of meloxicam, the presence of iron, appeared to modify the binding curve of each compound to DNA. A summary of the obtained stoichiometry in presence or absence of iron is shown in Table (2).

Table (2) shows that the presence of iron decreases the number of base pairs that are required for the binding of one molecule of diclofenac i.e. higher number of diclofenac molecules would be bound to the overall DNA helix. This effect was shown, to lesser extent for mefenamic acid and indomethacin, but absolutely not obvious for naproxen and meloxicam. It was interesting to observe that the influence of ferric ions on the binding stoichiometry was most seen with diclofenac, least observed with naproxen and meloxicam, whereas indomethacin and mefenamic acid lied in between, which correlates well with the observed effect of iron on the cytotoxicity of NSAIDs (Fig. 4). Overall, these observations further support the suggestion that NSAIDs-metal complexes by themselves possess cytotoxic activities (through binding to DNA and interfering with its normal functions, as one potential mechanism) rather than having a cytotoxic effect through depletion of vital metal ions.

CONCLUSIONS

All tested drugs were shown to bind to ferric ions with a stoichiometry of two drug molecules for each metal atom. The estimated binding constants appeared reasonably high (10^9-10^{14}) which suggests that the drug-complexes are maintained at physiological conditions.

Results indicated the presence of a correlation and, to a certain extent, a synergistic effect between the formation constant of the iron-drug chelates and the cytotoxicity of different ferric-NSAIDs against the MCF-7 cells. These imply that the ferric-NSAIDs- complexes possess cytotoxic activity by themselves, which is contrary to the common beliefs that chelating agents exert their cytotoxic effects through depletion of essential metal ions. Furthermore, ferric-NSAIDs complexes were shown to bind to DNA, though with slightly different ratio. These findings might help targeting NSAIDs towards tumor tissues where levels of metal ions such as iron has been shown to be significantly elevated compared to normal ones.

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Table 1: Formation constants and drug: metal stoichiometry determined for the drugs under investigation.

Complex	Stoichiometery (Drug: metal)	Estimated formation constant
Mefenamic acid: Fe^{3+}	2	2.40×10^{14}
Diclofenac: Fe^{3+}	2	1.00×10^{10}
Indomethacin: Fe^{3+}	2	1.29×10^{11}
Naproxen: Fe^{3+}	2	8.55×10^{9}
Meloxicam: Fe^{3+}	2	7.46×10^{11}

Table 2: Number of DNA bases that bind to one drug molecule in presence and absence of iron (r =2).

	Drug alone	Drug with Ferric ions
Diclofenac	6 and 2	Only 2
Mefenamic acid	14 and 4	10 and 4
Indomethacin	6 and 4	Only 4
Naproxen	4 and 2	4 and 2
Meloxicam	Only 10	Only 10

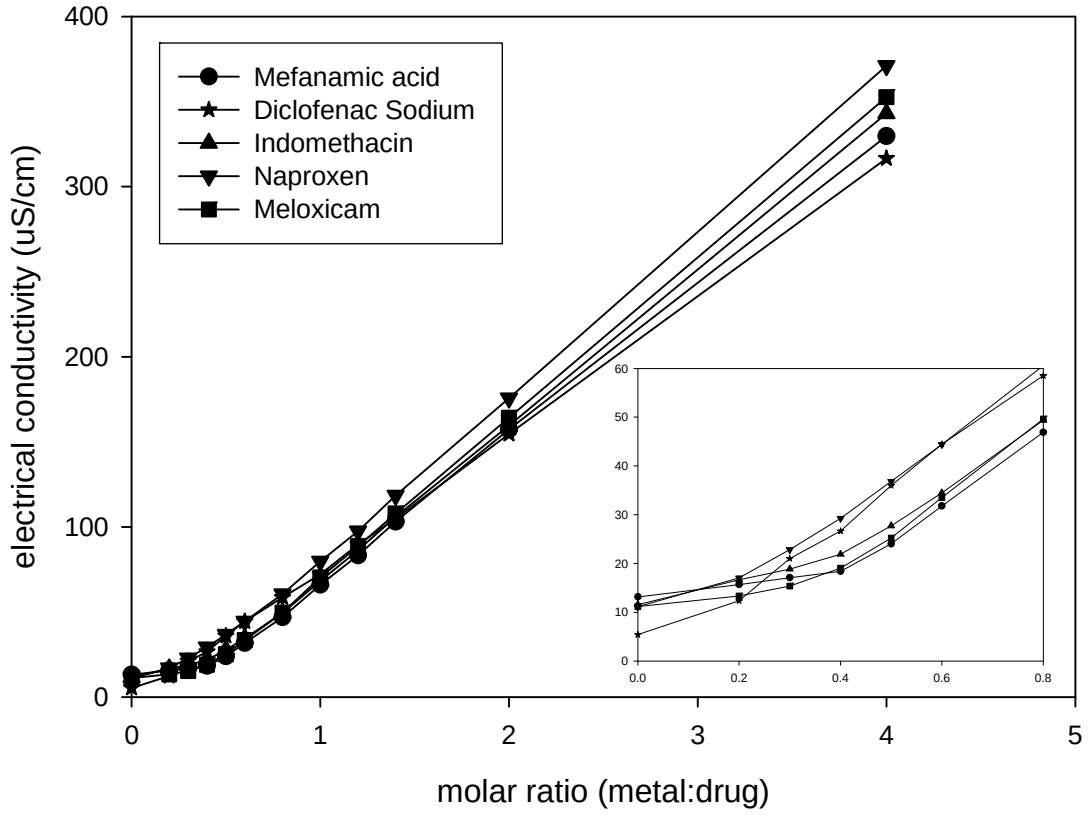


Figure 1. Molar ratio plots for the five NSAIDs under investigation with ferric ion. Results present the average of at least three determinations. Note the change in the slope of the curves at about r = 0.5 which supports a stoichiometry of 2:1. The error in measurements was generally less than 2%.

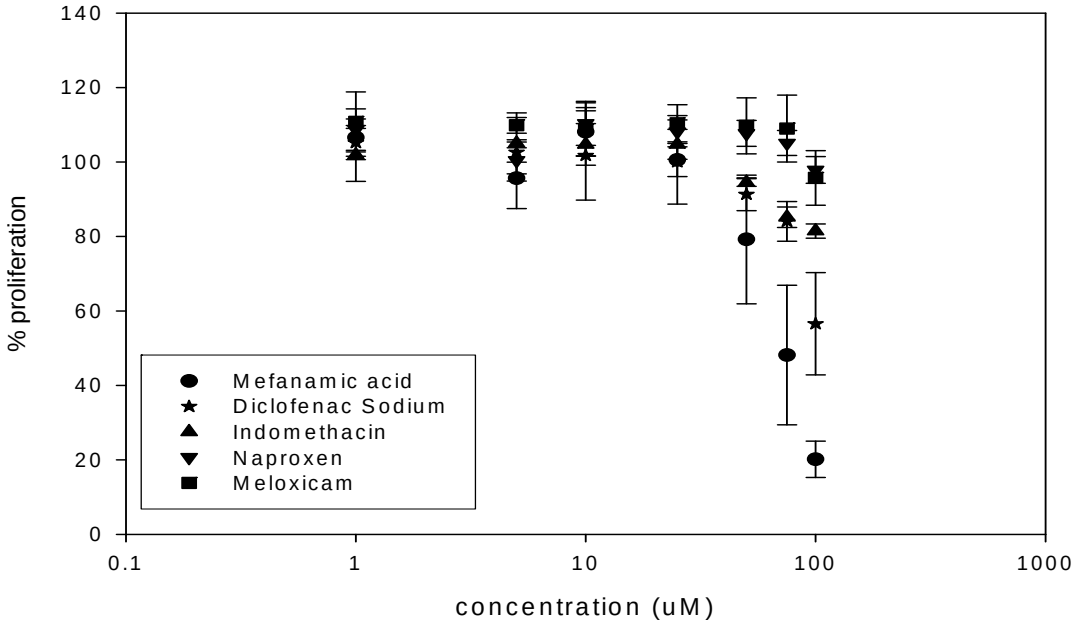


Figure 2. Antiproliferative activity of the NSAIDs under investigation against MCF-7 cells after an incubation of 72 hours. Results present the average and standard deviation of at least four replicates repeated on two different passage numbers.

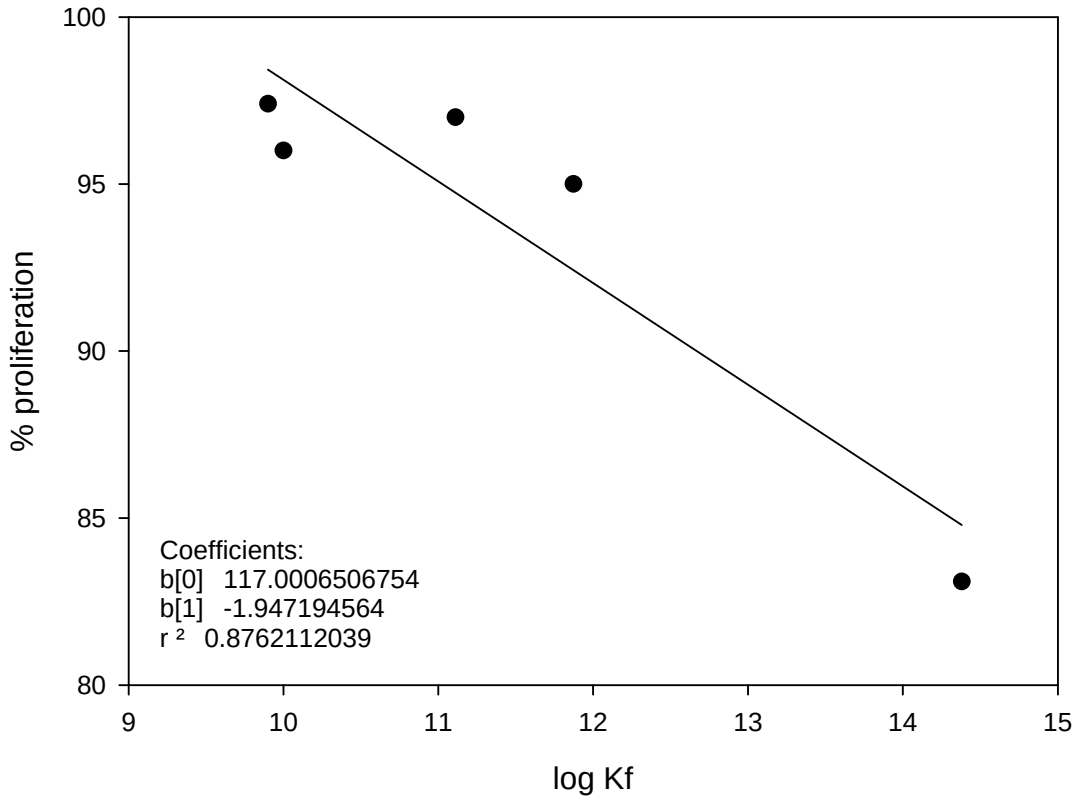


Figure 3. Correlation between the natural logarithm of the formation constant and percent proliferation (percent living cells) after 6 hours of incubation with the corresponding NSAIDs. Results present the average of at least four replicates for the proliferation assay.

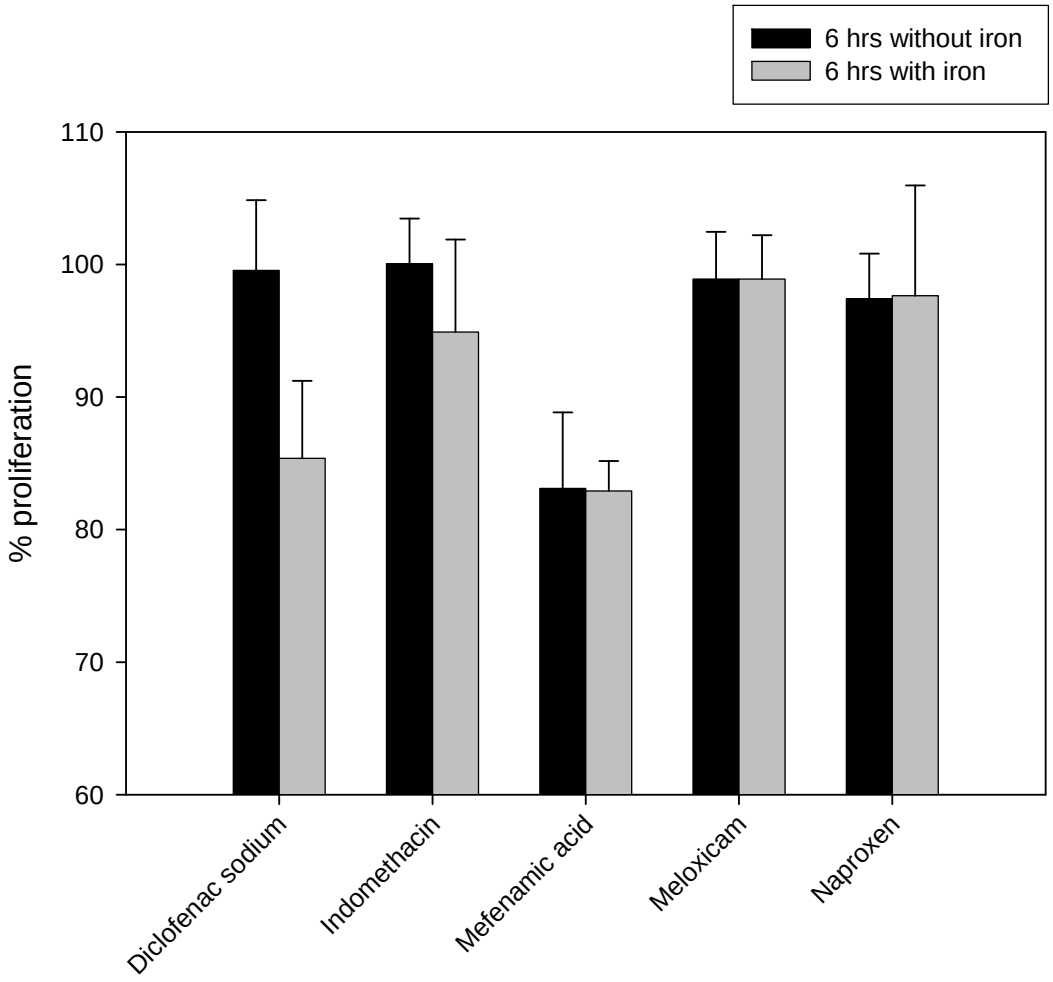


Figure 4. Comparison of the antiproliferative activity of the NSAIDs under testing with and without the presence of ferric ions in the supernatant of growing cells. Ferric ions were added two hours before the addition of the drugs and then incubation for 6 hours. Results represent the average and standard deviation of at least four replicates.

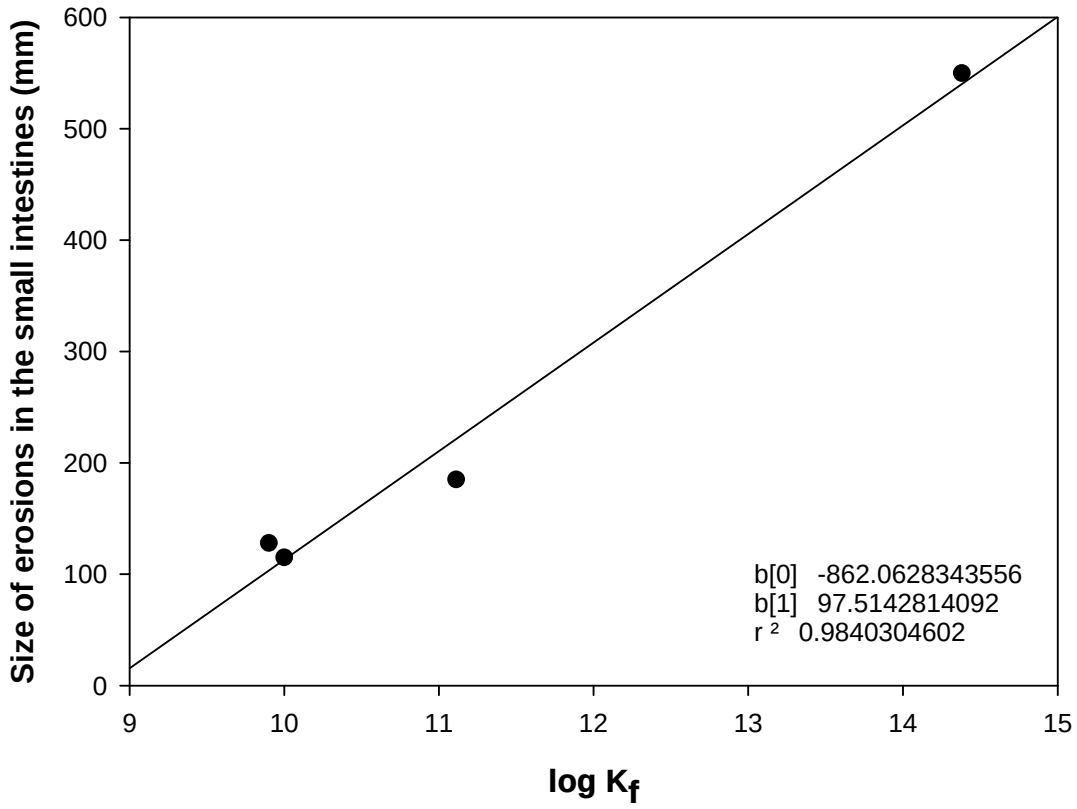


Figure 5. Plot of the size of lesions in the small intestines after treatment with NSAIDs against the calculated natural logarithm of formation constants. Values of the intestinal lesions are obtained from reference 40.

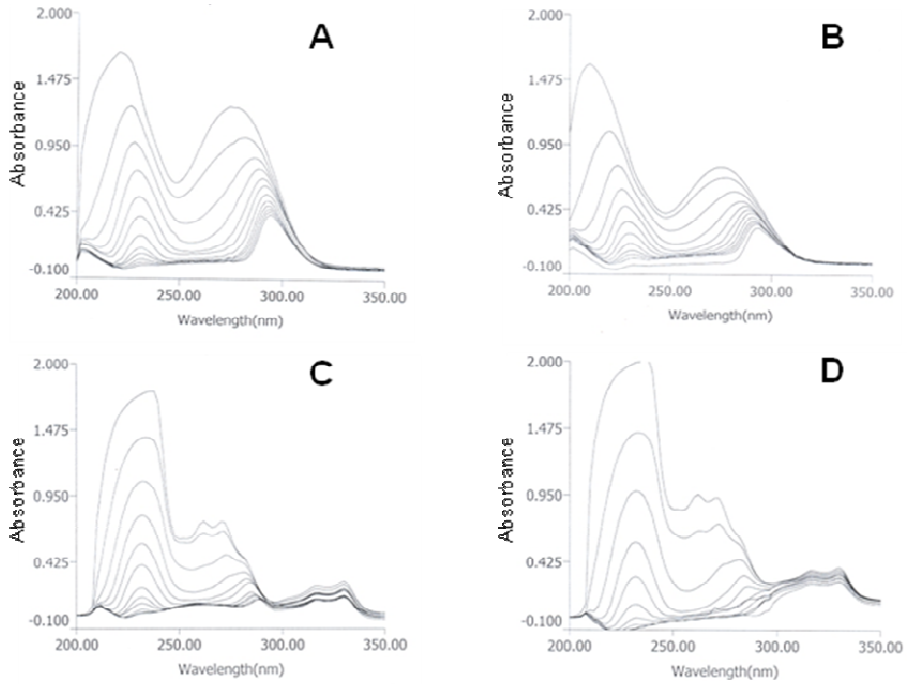
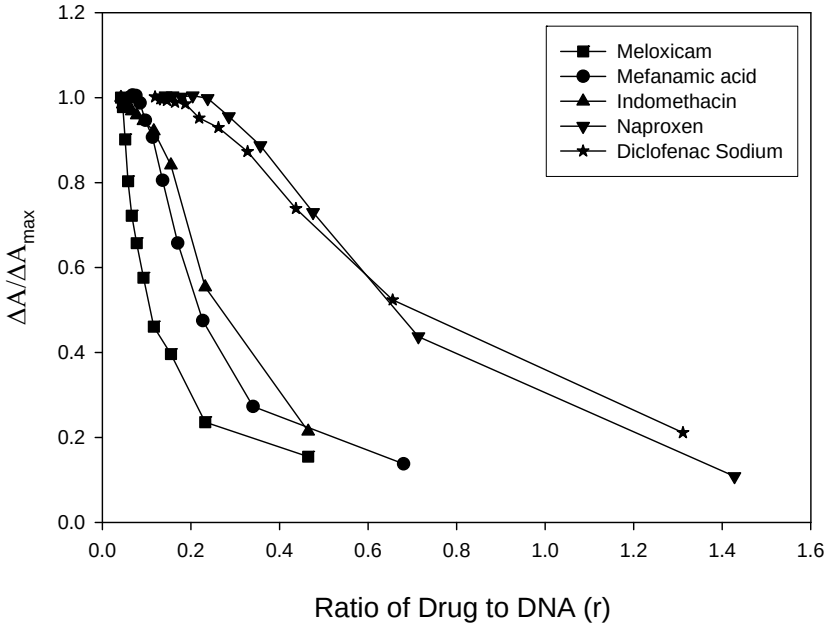


Figure 6. Representative overlaid UV spectra for the titration of diclofenac without (A) and with (B) ferric ions, and naproxen without (C) and with (D) ferric ions.

A)



B)

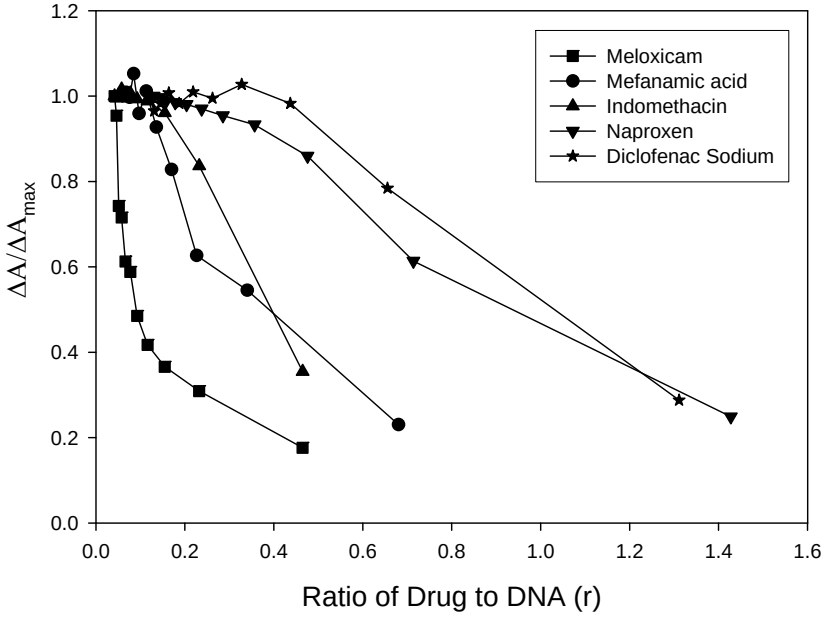


Figure 7. Binding curves for each of the tested compounds in absence (A) and presence (B) of ferric ions.

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قدرة خمسة من مضادات الالتهاب اللاستيرويدية على تكوين معقدات مع ايون الحديد

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ملخص

تمت دراسة قدرة خمسة من مضادات الإلتهاب اللاستيرويدية على تكوين معقدات مع أيون الحديديك (Fe^{3+}) وكذلك تم البحث في دور هذه المعقدات في تقليل نمو الخلايا السرطانية. وتم تحديد النسب المولية للارتباط (ووجدت 2:1 للمركبات جميعها) وثابت التكوين للمعقدات باستخدام طريقة التوصيل الكهربائي وقد تراوحت بين 10^{9} و10^{14}. تم اختبار فاعلية المركبات الخمسة كمواد مثبطة للنمو بوجود أيون الحديديك وغيابه، وذلك باستعمال الخلايا من نوع (MCF-7). ووجد أن حمض ميفانيميك والديكلوفيناك يتمتعان بأعلى فاعلية بعد 72 ساعة، حيث بلغت التراكيز المثبطة لنمو نصف الخلايا 15.29 ± 70.54 µM و 11.28 ± 108.38 µM لكل من حمض ميفانيميك والديكلوفيناك على الترتيب. تراوحت فاعليات المركبات : إندوميثاسين، نابروكسين و ميلوكسيكام بين المنعدمة والمتوسطة. وتبين وجود علاقة خطية ذات ثابت ارتباط = 0.876 بين ثابت التكوين للمعقدات وقدرتها على قتل الخلايا على زمن 6 ساعات. وتم فحص قابلية كل دواء على الارتباط بالحمض النووي ال DNA بوجود أيون الحديديك وغيابه، وقد تبين أن المعقد يرتبط بالحمض النووي، ولكن مع بعض التغيير في نسبة الارتباط، وتشير النتائج إلى أن المعقدات تتمتع بقدرة ذاتية على قتل الخلايا.

الكلمات الدالة: الفعالية المثبطة للنمو، التمخلب، ثابت التكوين، خلايا MCF-7 ، الحمض النووي DNA ، مضادات الالتهاب اللاستيرويدية

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