

EVALUATING THE IN VITRO BIOLOGICAL EFFICACY OF NOVEL KETOPROFEN HYBRIDS AGAINST INFLAMMATION, ARTHRITIS, AND OXIDATIVE STRESS

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Abstract

In this study, we disclose the outcomes of the biological assessment of recently synthesized hybrids of ketoprofen with 2-phenylethylamines and 1,2,3,4-tetrahydroisoquinolines. These hybrids were obtained using an environmentally friendly method that minimizes the generation of waste products. The newly acquired hybrids were analysed to evaluate their antioxidant, anti-inflammatory, and anti-arthritic activities. Additionally, as potential drugs exhibiting favourable results, their experimental partition coefficient in a biphasic system of lipids and water (logP) was determined.

Key words: ketoprofen, hybrid molecules, HPSA, IAD, ATA, in vitro, in silico

Introduction. Ketoprofen, a potent non-steroidal anti-inflammatory drug (NSAID) belonging to the class of 2-arylpropionic acid, has garnered extensive clinical use all over the world for many years. While its efficacy in pain management is well established, its short terminal half-life presents challenges in maintaining therapeutic levels. Prolonged-release formulations have been developed to

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address this limitation, aiming to extend the duration of action and reduce gastrointestinal side effects. Despite these advancements, gaps persist in our understanding of ketoprofen's pharmacokinetics, including its stereoselective binding, metabolic pathways, and interactions with other drugs. Beyond addressing pharmacokinetic limitations, exploring the antioxidant, anti-inflammatory, and anti-arthritic activities of the new ketoprofen derivatives holds significant promise. The preliminary in vitro assays offer valuable insights into the biological characteristics of the newly synthesized ketoprofen derivatives. This will contribute to the understanding of the therapeutic potential and pharmacological profile of these derivatives, aiding in their further development as promising candidates for inflammatory disorders.

Through the intramolecular α -amidoalkylation reaction of various ketoprofen amides using a green catalyst (triflic acid absorbed on silica, TfOH/SiO₂), we have successfully synthesized a series of ketoprofen hybrids with 2-phenylethylamines and 1,2,3,4-tetrahydroisoquinoline (Fig. 1) [1].

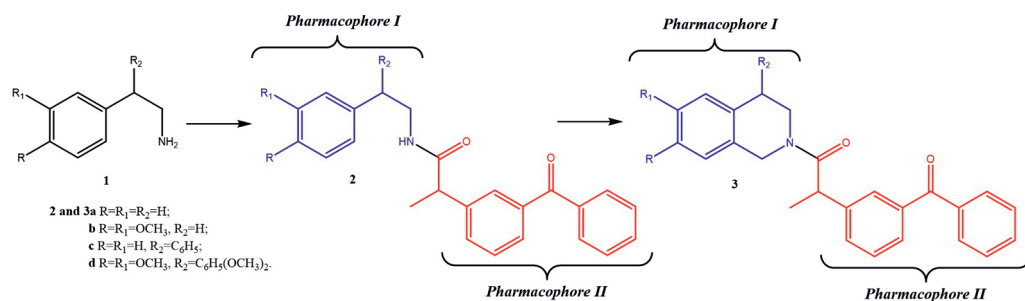


Fig. 1. Synthesis of new ketoprofen hybrids [1]

In the present investigation, our focus was on examining the impact of substituents within the structure of pharmacophore I, with no structural alterations made to pharmacophore II. Given the established properties of ketoprofen and its incorporation into the structure of novel molecules, we deemed it suitable to adopt ketoprofen as a benchmark for comparing the activities of the newly developed ketoprofen hybrid molecules.

Experimental. In the present work, we investigated antioxidant activity, in vitro anti-inflammatory, and anti-arthritic activity. The antioxidant activity was evaluated as hydrogen peroxide scavenging activities (HPSA) [2].

In vitro analysis of anti-inflammatory activity was assessed as inhibition of albumin denaturation (IAD). The analysis was performed according to Manolov method [3].

Anti-tryptic activity (ATA) is known also as an in vitro anti-arthritic activity. The analysis was performed according to the method of OYEDAPO and FEMUREWA [4] with minor modification as described by MANOLOV et al. [3].

Results and discussion. All ketoprofen derivatives synthesized underwent in vitro assessments for their inhibition of albumin denaturation (IAD), antioxidant activity, and anti-tryptic activity (ATA). The in vitro findings are juxtaposed with in silico predictions, and a detailed presentation of the study results is provided in Table 1.

T a b l e 1
In vitro and in silico results of the conducted biological activity

Compound	IC ₅₀ ± SD, µg/mL			R _M ± SD	Pa	
	HPSA	IAD	ATA		cAnti-I	cAnti-A
AA	24.84 ± 0.35	—	—	—	—	—
Qrc	69.25 ± 1.82	—	—	—	—	—
Ibu	—	81.50 ± 4.95	259.82 ± 9.14	1.11 ± 0.010	0.903	0.573
Ket	—	345.56 ± 41.81	720.57 ± 19.78	1.54 ± 0.015	0.925	0.469
2a	84.78 ± 2.05	58.44 ± 0.77	175.78 ± 10.68	2.25 ± 0.015	0.490	0.227
2b	70.64 ± 0.75	58.59 ± 0.91	117.02 ± 6.71	1.30 ± 0.015	0.442	0.268
2c	67.28 ± 1.21	81.09 ± 0.79	116.09 ± 4.08	1.55 ± 0.023	0.450	—
2d	69.28 ± 1.11	58.83 ± 0.62	136.07 ± 9.81	1.65 ± 0.016	0.433	0.266
3a	76.58 ± 1.57	72.28 ± 0.42	230.91 ± 12.97	1.77 ± 0.015	0.604	—
3b	69.65 ± 1.06	60.66 ± 0.49	143.22 ± 10.71	1.41 ± 0.011	0.585	—
3c	62.57 ± 1.99	79.76 ± 0.53	103.43 ± 7.50	1.39 ± 0.010	0.603	—
3d	64.05 ± 1.12	71.61 ± 0.73	122.48 ± 10.41	1.93 ± 0.012	0.605	—

The results for antioxidant activity (HPSA), inhibition of albumin denaturation (IAD) and anti-tryptic activity (ATA) are presented as IC₅₀. Ascorbic acid (AA), quercetin (Qrc), ibuprofen (Ibu) and ketoprofen (Ket) were used as a standard. R_M is dimensionless because it is a function of R_f and is determined by thin layer chromatography. cAnti-I and cAnti-A are expressed as P_a (probability of being “active”) and calculates the probability that a test compound belongs to a subclass of active compounds (similar to the molecule structures that are most typical of a subset of “assets” in PASS training kit). The value of the most active compound is 1. R_M – lipophilicity; cAnti-I – the predicted anti-inflammatory activity made with PASS; cAnti-A – the predicted anti-arthritis activity produced by PASS

Antioxidant activity (HPSA). Reactive oxygen species (ROS) are chemically reactive oxygen containing radicals and molecules [super oxide (O₂[−]), hydroxy (OH), peroxy (ROO) and alkoxy (RO), HOCl, ozone (O₃), peroxyxynitrite (ONOO[−]), singlet oxygen (¹O₂) and H₂O₂]. They are naturally formed as a by-product of cellular metabolism. Under physiological conditions, enzyme systems regulate the ROS levels. They are known to damage vital molecules of significant biological importance such as phospholipids, proteins, and DNA. The damage caused by them has been shown to be involved in the development of a number of diseases (cancer, cardiovascular disease, atherosclerosis, and Alzheimer’s disease) [5]. Even in a state of physiological health, the toxic effects of oxygen and its derivatives, accumulating in the body, lead to a shortening of life expectancy [6]. In the present work the research was focused on the deactivation of hydrogen peroxide. Hydrogen peroxide is an oxidant that is continuously formed

in living tissues as a result of several metabolic processes. But its detoxification is very important in preventing it from entering into harmful reactions, such as the Fenton reaction. H_2O_2 reacts with iron ions (Fe^{2+}), generating extremely reactive and harmful hydroxyl radicals (OH) [7]. The results obtained for the antioxidant activity of the synthetic analogues of ketoprofen were compared with ascorbic acid and quercetin as a standard. They are natural compounds and have proven antioxidant properties. The antioxidant activity values of the synthesized hybrids ranged from 62.57 $\mu\text{g/mL}$ to 84.78 $\mu\text{g/mL}$ (Table 1).

Compared to ascorbic acid (24.84 $\mu\text{g/mL}$), the synthesized ketoprofen analogues show low antioxidant activity, but compared to quercetin 4-aryl substituted hybrids **3c** and **3d** show higher activity and this trend is observed in their precursors **2c** and **2d**.

Tetrahydroisoquinolines containing in the 4th position a benzene nucleus are structural analogues of the alkaloid cherylline, which exhibits a vast number of biological activities. Compounds **2a** and **3a** show low activity.

The inflammatory process also causes and accelerates the formation of ROS. In particular, the formation of a superoxide anionic radical at the site of inflammation and this is associated with the formation of other ROS species, such as H_2O_2 . It is also involved in reductive decomposition reactions of hydrogen peroxide (the so-called Haber–Weiss reaction) and organic hydroperoxides ROOH, assuming that at least some of the oxygen produced in these reactions is in a singlet state [8,9]. For this reason, the removal of H_2O_2 is very important to prevent the generation of OH radicals.

Inhibition of albumin denaturation. Inflammation is the reaction of living tissues to injury. It involves a complex set of enzymatically activated reactions, mediator release, fluid extravasation, cell migration, tissue degradation, and repair [10]. Protein denaturation is also a well-documented cause of inflammation in rheumatoid arthritis. Several anti-inflammatory drugs have shown a dose-dependent ability to thermally inhibit protein denaturation [11]. Synthetic analogues of ketoprofen have been studied to inhibit albumin denaturation. This method provides information on the extent to which albumin can be protected from denaturation when heated. We used human albumin for this purpose. The percentages of inhibition of the various ketoprofen hybrids are presented in Table 1. The results of the study are presented as IC_{50} . Since ibuprofen and ketoprofen have proven anti-inflammatory properties, we used them as standards for comparing the activity of the newly synthesized analogues of ketoprofen. The IC_{50} of ibuprofen and ketoprofen, estimated as IAD, was 81.50 $\mu\text{g/mL}$ and 345.56 $\mu\text{g/mL}$, respectively (Table 1). The results obtained show that the IC_{50} of the hybrids ranged from 58.44 $\mu\text{g/mL}$ to 81.09 $\mu\text{g/mL}$ (Table 1). The analysis shows that ketoprofen-amides **2a**, **2b**, and **2d** (58 $\mu\text{g/mL}$) as well as cyclic ketoprofen **3b** (60 $\mu\text{g/mL}$) are characterized by higher activity than both ibuprofen and other hybrid molecules.

Comparing the activity between amides (**2a-d**) and cyclic (**3a-d**) ketoprofen derivatives, we found out that the amides show a higher degree of stabilization of the albumin molecule from denaturation upon heating. In general, ketoprofen hybrids are characterized by good IAD compared to ketoprofen. In this case, their activity is five times higher than that of ketoprofen (Table 1). They are also more active than ibuprofen. This fact is explained by the presence of a benzene nucleus in their structure and for this reason they have the ability to allosterically bind to albumin. Apparently, this is the reason for the high stabilization of albumin from denaturation when heated. Human serum albumin is known to have two major allosteric binding sites for exogenous active substances: Sudlow I and Sudlow II. According to the Sudlow nomenclature, FASANO et al. [12] found that bulk heterocyclic molecules bind to Sudlow I (located in subdomain IIA), while aromatic carboxylic acids and profens bind to Sudlow II (located in subdomain IIIA). We hypothesize that ketoprofen hybrids bind to Sudlow I (Fig. 2). Further research in this area is forthcoming.

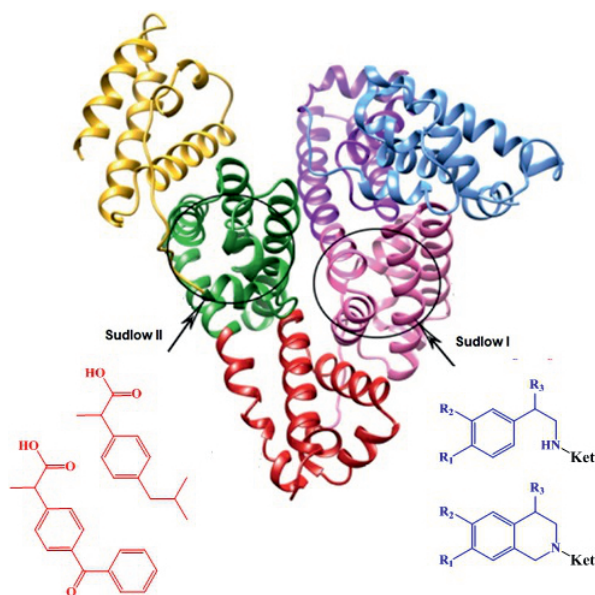


Fig. 2. Allosteric centres of human serum albumin
Sudlow I and Sudlow II

Anti-tryptic activity. Proteinases have been shown to cause arthritis. Neutrophils are known to be a rich source of proteinases, which contain a large number of serine proteinases in their lysosomal granules. Proteinase contained in leukocytes is known to play an important role in tissue damage during inflammatory reactions, and a significant level of protection is provided by proteinase inhibitors [4,11]. In vitro anti-arthritic activity has been evaluated as anti-tryptic activity [4]. The aim of this study was to inhibit the enzyme trypsin, which is

also a group of serine proteinases. In the present work, we investigated the inhibition of the enzyme trypsin. The newly synthesized ketoprofen hybrids were scanned for ATA (Table 1). IC₅₀ results for ATA ranged from 103.43 µg/mL to 230.91 µg/mL. From the analysis, we found that profens (ibuprofen and ketoprofen) show a higher IAD than ATA. The presence of a benzene nucleus in the structure of ketoprofen hybrids significantly increases the activity. Compounds **3c**, **2c**, **2b**, **3d**, **2d**, **3b**, **2a**, and **3a** show higher ATA compared to ibuprofen and ketoprofen (Table 1). The data from the analysis show that the activity of the synthetic analogues of ketoprofen depends on the presence of aryl and methoxy substituents, a dependence established by our previous studies [3].

Of the amides, **2c** show the highest activity, and of the cyclic compounds – **3c** (Table 1).

In general, the results of in vitro studies show that ketoprofen hybrids exhibit IAD and ATA. From both experiments we can derive important information about the properties of potential new drugs and both experiments are related to the preservation of the integrity of the albumin molecule.

Experimental determination of lipophilicity (R_M). Lipophilicity is extremely important for all drug “candidates”. It helps scientists predict and also better understand the passage of drugs into physiological systems. It plays an extremely important role in both the pharmaceutical and biotechnology industries. Lipophilicity is a determining factor in evaluating the so-called ADME properties of each new drug, namely in the absorption of the compound, distribution in the body, passage through various membranes and biological barriers, metabolism, and excretion (ADME – Absorption, Distribution, Metabolism, and Excretion).

Lipophilicity can be both calculated and determined experimentally. Lipophilicity was experimentally determined on the resulting ketoprofen hybrids using reverse phase thin layer chromatography, a method reported by PONTIKI and HADJIPAVLOU-LITINA [13]. Table 1 shows the results obtained. It is seen that hybrids **2a**, **3a**, and **3d** are the most lipophilic. They are also more lipophilic than ketoprofen and ibuprofen. The others have lipophilicity close to that of ketoprofen and higher than that of ibuprofen.

Conclusion. In summary, synthesized ketoprofen analogues, notably 4-aryl hybrids **3c** and **3d**, display superior antioxidant activity, surpassing quercetin. Tetrahydroisoquinolines, resembling cherylline, show varied biological activities. In anti-inflammatory research, ketoprofen-amides **2a**, **2b**, **2d**, and cyclic ketoprofen **3b** exhibit enhanced efficacy compared to ibuprofen and ketoprofen. Amides (**2a-d**) demonstrate superior albumin stabilization, with ketoprofen hybrids showing fivefold higher inhibitory activity (IAD) than ketoprofen alone. The benzene nucleus enables allosteric binding, suggesting their potential as dual inhibitors. In arthritis research, especially **3c** and **2c**, ketoprofen hybrids demonstrate significant anti-tryptic activity (ATA), emphasizing their potential as dual inhibitors and offering insights for drug development while preserving albumin integrity.

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