

Review Article

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Non-Antibiotic Compounds: The Activity of the NSAID Diclofenac on Bacteria- A Review

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ABSTRACT

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Bacterial multidrug resistance is a phenomenon that has been growing with increasing speed worldwide. New mechanisms of resistance have been described in different bacterial pathogens, representing a threat to effective treatments of common infectious diseases with currently available drugs. The use of drugs known as "non-antibiotics" are among the alternative approaches to the use of conventional antibiotics. This approach has been studied in order to counteract the global threat of multidrug resistance. This group of drugs includes various non-steroidal anti-inflammatory drugs (NSAIDs). The NSAID diclofenac has demonstrated broad-spectrum antimicrobial activity *in vitro* and *in vivo*. In addition, this drug has a synergistic antibacterial effect when combined with certain other drugs. It may also show anti-biofilm activity, and may inhibit or decrease the activity of certain virulence factors in some bacterial pathogens.

Introduction

In recent decades, due to the dramatic increase and global spread of bacterial resistance to a number of commonly used antibacterial agents, many studies have been directed at investigating drugs whose primary therapeutic purpose is not antimicrobial action.

Classic commonly used antimicrobials include antibiotics of natural origin produced by fungi and bacteria (e.g., penicillin G and streptomycin), semi-synthetic antibiotics (e.g., oxacillin and amoxicillin), and drugs produced entirely via chemical synthesis, such as quinolones and sulphonamides. However,

studies have shown that certain other pharmacological classes of drugs – such as neuroleptics (Kristiansen, 1979), antihistamines (Dastidar *et al.*, 1996), and non-steroidal anti-inflammatory drugs (NSAIDs) (Zimmermann and Curtis, 2017; Shah *et al.*, 2018) – have a greater or lesser degree of broad-spectrum antibacterial activity. Kristiansen (1992) coined the term “non-antibiotics” for such drugs.

Among these "non-antibiotics", most studies have focused on NSAIDs, drugs that – in addition to anti-inflammatory action – have analgesic and antipyretic properties. These drugs are among the most widely used in the

world. Their main mechanism of action involves the inhibition of cyclooxygenase, which leads to a decrease in the synthesis of prostaglandins (Dinarello, 2010).

The most commonly prescribed NSAIDs in clinical practice include drugs such as diclofenac, ibuprofen, indomethacin, and acetylsalicylic acid. Their antimicrobial action comprises bacterial isolates of different species, as well as fungi (Zimmermann and Curtis, 2017).

Diclofenac is the 2- (2,6-dichloranilino) phenylacetic acid. It can be supplied as either sodium or potassium salt, both with good solubility in solvents such as methanol and DMSO. However, most studies involving the action of diclofenac on bacteria have been conducted using its sodium form. This NSAID showed antimicrobial activity in vitro against different bacterial pathogens (Tables 1 and 2). In an interesting approach, Alqahtani *et al.*, (2018) prepared chitosan nanoparticles loaded with diclofenac and demonstrated high activity of this cluster against *S. aureus* and *B. subtilis*, which depended on the molecular weight of chitosan and pH.

The mechanism of action of diclofenac seems to reside mainly in the inhibition of DNA synthesis (Dastidar *et al.*, 2000). Particularly in *E. coli*, some NSAIDs showed an ability to inhibit the DNA polymerase III β subunit. The change of this subunit as a consequence of the binding of the NSAID molecule results in inhibition of DNA replication and repair (Yin *et al.*, 2014). Besides that, the increase in the uptake of ethidium bromide in *S. aureus* cells exposed to diclofenac sodium has provided evidence that NSAIDs may also act by compromising cellular membrane integrity (El-Baky and El-Gendy, 2016).

The activity of diclofenac has been reported as bactericidal for Gram-positive and Gram-

negative bacteria, in addition to mycobacteria (Dastidar *et al.*, 2000; Dutta *et al.*, 2007a, 2007b; Mazumdar *et al.*, 2009). However, Perilli (2000) showed that the action of this drug on the growth of *S. epidermidis* was bacteriostatic, since subsequent subcultures restored normal growth rates of the isolates.

Riordan *et al.*, (2011) described extensive changes in the transcriptome of methicillin-resistant *S. aureus* (MRSA) strain COL when grown with subinhibitory concentrations of diclofenac. Changes in the expression of hundreds of genes were noted, including those associated with resistance to antimicrobials. Transcriptional alterations were measured using gene expression microarrays and real-time quantitative polymerase chain reactions (qPCR). It was further shown that diclofenac altered susceptibility to multiple antibiotics in a strain-dependent manner. Susceptibility increased for ciprofloxacin, ofloxacin, and norfloxacin, decreased for oxacillin and vancomycin, and did not change for tetracycline or chloramphenicol.

In *E. coli* isolates of urinary tract infections, diclofenac exhibited pronounced antibacterial activity; sequential exposure to high concentrations of the drug resulted in the emergence of mutants with noticeable reduction in the minimum inhibitory concentration (MIC) values of the antibiotics. In addition, agarose gel electrophoresis showed the absence of any specific band of plasmid in the mutant, in contrast to what was observed in the control isolate (Mazumdar *et al.*, 2006).

Studies have shown that the broad-spectrum antibacterial effects promoted by NSAIDs are not restricted to the results obtained in vitro. Administration of diclofenac to mice at doses of 1.5 and 3.0 $\mu\text{g} / \text{g}$ of body weight could significantly protect these animals from death after experimental infection with *S. enterica*

ser. *typhimurium* (Annadurai *et al.*, 1998). Identical results were obtained by Dutta *et al.*, (2007a).

Diclofenac was also able to act in experimental infections caused by *M. tuberculosis* and *Listeria monocytogenes*. The inoculation of *M. tuberculosis* in mice followed by treatment with diclofenac resulted in fewer lesions and lower bacillary loads than were found in control animals (Dutta *et al.*, 2004a). A similar finding was reported by Dutta *et al.*, (2004b), with a marked decrease in the lesions of organs of mice treated with diclofenac after infection with *M. tuberculosis*.

Dutta *et al.*, (2008b) also showed that diclofenac provided protection to BALB/c mice challenged orally with *L. monocytogenes*. Significant reduction of bacterial counts in the liver and spleen, a 10-fold decrease in the number and size of necrotic foci, and the positive regulation of inflammatory cytokine expression were observed in this study.

Synergy with antimicrobial drugs

Besides the direct antimicrobial action of NSAIDs, their association with antibiotics can result in inhibitory or bactericidal synergistic activity against bacterial pathogens. Annadurai *et al.*, (2002) found clear in vitro synergism between diclofenac and streptomycin for different species of Gram-positive and Gram-negative bacteria. In vivo, administration of both drugs in mice previously infected with *Salmonella* resulted in notable reductions in the number of pathogens found in blood, liver, and spleen samples.

Using a disk diffusion test, Dutta *et al.*, (2007a) found that combining diclofenac with streptomycin resulted in significant increases

in the zones of inhibition of *E. coli* ATCC 25922. Likewise, as reported by Annadurai *et al.*, (2002), the association of these drugs resulted in a significant reduction in *Salmonella* counts in the organs of infected mice. Heightened inhibition was also shown in vitro for *M. smegmatis* (Dutta *et al.*, 2004b), and this synergism was later corroborated in vivo: mice intravenously infected with *M. tuberculosis* and then treated with these drugs showed a notable decrease in the count of bacteria recovered from the lungs and spleen, compared with mice receiving streptomycin alone (Dutta *et al.*, 2007b).

A synergistic effect was still observed for *Listeria monocytogenes* in invitro tests using diclofenacin association with gentamicin (Dutta, Mazumdar and Park, 2009). On the other hand, when Shepherd *et al.*, (1998) compared the efficacy and safety of ocular drops containing a combination of diclofenac and gentamicin and eyedrops containing only gentamicin – both administered during the postoperative management of patients undergoing cataract surgery and lens implantation – they concluded that the combined drugs were more effective in controlling inflammation.

Diclofenac and biofilms

In addition to investigating their potential for antibacterial activity, researchers have analyzed the effects of NSAIDs on biofilms and other factors linked to bacterial virulence. Biofilms are complex multicellular communities of microorganisms that proliferate while surrounded by a polymeric matrix, conferring an antimicrobial survival capacity and protection against the immune defense mechanisms of the host (Otto, 2013)

The mechanism by which NSAIDs impact biofilm formation is not completely understood. The reduction of components that

may form part of the biofilm matrix (such as extracellular polysaccharides, teichoic acids and proteins), alteration of cell surface hydrophobicity, or inhibition of components of the quorum-sensing system are factors that must be considered (Oprea and Moga, 2015).

In most studies of drugs with potential for anti-biofilm activity, the objective is to evaluate the effect on preformed biofilms. This approach depends on the drug's ability to diffuse through the biofilm to exert its effect directly on the pathogen, or to act by disintegrating the biofilm so that the exposed pathogen becomes an easier target for antimicrobials. In other studies, however, the investigative focus is on the drug's ability to act during the stages during which planktonic cells adhere to a surface, in order to inhibit the early stages of biofilm formation.

According to Hegazy (2016), subinhibitory concentrations of diclofenac resulted in marked inhibition of biofilm formation produced by *P. mirabilis* (90% in $\frac{1}{2}$ MIC), and decreased the value of the MBIC (minimum biofilm inhibitory concentration) for the different antibiotics tested. Primary bacterial adhesion was not significantly affected, indicating that effective drug activity occurred during the later stages of biofilm formation. As reported by Hegazy (2016), this may be the result of diclofenac's effect on the intercellular signaling system known as quorum sensing. This system is based on cell density and regulates functions that contribute to the virulence of many bacterial pathogens, including biofilm production (Waters and Bassler, 2005).

El-Baky and El-Gendy (2016) also reported that different NSAIDs produced a noticeable inhibitory effect on both biofilm formation and preformed *S. aureus* biofilm. While meloxicam was identified as the NSAID with the greatest capacity to inhibit biofilm

formation, diclofenac was found to have the greatest ability to degrade preformed biofilm. Both effects were higher than those observed for the antimicrobial levofloxacin. This study further revealed that NSAIDs negatively regulated *icaA* gene expression. This gene is one of the components of the *icaADBC* operon, which encodes the production of PIA (polysaccharide intercellular adhesin), the main component of the extracellular matrix of the polysaccharide biofilm in *Staphylococcus* (Cramton *et al.*, 1999).

The effect of NSAIDs on biofilm may vary according to the particular characteristics of strains of a species. Perilli (2000) investigated two slime-positive isolates of *S. epidermidis* from catheter-associated infections: one negative and one positive for a slime-associated antigen (SAA). In the SAA-negative strain, short-term treatments with diclofenac significantly reduced biofilm production in microtiter plates; the SAA-positive strain was not affected. After the colonization and treatment of each strain on a contact lens surface, scanning electron microscopy confirmed that the drug promoted complete disorganization of the biofilm structure in the SAA-negative strain. The drug's lack of effect on the SAA-positive strain was attributed to the differences between the composition of its slime material and that of the SAA-negative isolate.

Reśliński, Dąbrowiecki and Głowacka (2015) showed that diclofenac – in concentration equal to that reached in human serum – limited the formation of strong biofilm by clinical isolates of *S. aureus* and *E. coli* grown on the surface of polypropylene mesh, which is commonly used in hernia surgery. On the other hand, the percentage of strains which formed weak biofilm increased from 1.4 % (control regimen) to 10.0 % (diclofenac). In our research, we have also observed diclofenac's effect on the induction of weak

biofilm production in some *S. aureus* isolates (work in progress – Figure 1).

In a clinical strain of *P. aeruginosa* that produced a strong biofilm, diclofenac (1/4 of MIC) reduced biofilm formation to about 60% of that observed in the control (Abbas 2015). However, an earlier study showed that the activity of this drug on preformed biofilms by clinical strains of *P. aeruginosa* was only moderate.

The effects of combining diclofenac with other drugs on biofilm production

In some investigations, the focus was on assessing whether NSAIDs – combined with each other or associated with other substances – affect biofilm formation, or alter the density of preformed biofilms. Baldiris *et al.*, (2016) reported that the combination of diclofenac and ibuprofen in subinhibitory concentrations produced a significant reduction in biofilm formation in clinical isolates of *K. pneumoniae* and *E. coli*, both previously characterized as producers of strong biofilms. The association of diclofenac (and also ibuprofen) with N-acetyl cysteine resulted in an marked disruptive effect on mature biofilms formed by some Gram-negative bacteria and *S. aureus* (Mohsen *et al.*, 2015).

Increased anti-biofilm effect was also observed in the combination of diclofenac or ibuprofen with calcium paste (CH). Calcium hydroxide is widely used in dentistry because of its antimicrobial and biological effects. Combining these NSAIDs with CH did not interfere in the paste's pH, but did increase antimicrobial action against the biofilm of *Enterococcus faecalis* (de Freitas *et al.*, 2017). The authors suggested that the use of NSAIDs (such as diclofenac) in tandem with CH paste would be a possible alternative to antibiotic use.

The impact of diclofenac on lotic biofilms

NSAIDs such as diclofenac have potential toxicity, and their presence in the environment may have adverse impacts on fauna and microbiota. These drugs can be found in significant amounts in aquatic environments in many parts of the world (Lonappan *et al.*, 2016). Biofilms are considered important indicators of both the toxicity and degradation processes of chemical pollutants in aquatic environments, as these represent sites in which such chemicals interact with a diverse and complex microbiota (Sabater *et al.*, 2007). In this way, some studies have demonstrated the effect of diclofenac on lotic biofilms.

Paje *et al.*, (2002) used river water as the inoculum and sole source of nutrient supply for the growth of environmental biofilms with a wide variety of bacteria, cyanobacteria, and algae. The biofilm development of the control experiment was compared to that of diclofenac-treated reactors. After four weeks of exposure to 100 µg/L diclofenac, the older biofilms – grown for 11 weeks before exposure to diclofenac – lost up to 70% of their initial biomass.

On the other hand, bacteria from the *Cytophaga-Flavobacterium* group survived to exposure to diclofenac and were able to degrade more than 90% of the original compound within five days of its application, demonstrating the ability of some microorganisms to adapt to this compound. Additionally, the authors reported that diclofenac promoted a selective effect, reducing the proportion of the population of Gram-positive bacteria. About 68% of the colonies isolated from the untreated biofilms were Gram-positive while almost the same proportion of isolates from the diclofenac-treated reactors was Gram-negative bacteria.

Table.1 Diclofenac inhibitory activity against Gram negative bacteria and the methods employed

Bacteria	Source	MIC ($\mu\text{g/mL}$)	Method	Reference
<i>Escherichia coli</i>	Clinical	0.25 – 1024 [256]	Agar dilution	Ahmed <i>et al.</i> , (2017)
	Clinical	2 - 100	Broth dilution	Mazumdar <i>et al.</i> , (2006)
	ATCC 25922	10	Broth dilution	Mazumdaret <i>et al.</i> , (2006)
	Clinical	50 - >1000	Agar dilution	Duttaet <i>al.</i> , (2007a)
	K12 C600	50	Agar dilution	Duttaet <i>al.</i> , (2007a)
	Clinical	2500	Broth dilution	AL-Janabi <i>et al.</i> , (2009)
<i>Klebsiellapneumoniae</i>	ATCC 10031	1024	Agar dilution	Ahmed <i>et al.</i> , (2017)
	ATCC 10031	173.7	AWD with MIC*	Mohsen <i>et al.</i> , (2015)
<i>Klebsiellaspp</i>	Clinical	2 - 1024[256]	Agar dilution	Ahmed <i>et al.</i> , (2017)
	Clinical	≤ 200 - >100	Agar dilution	Dutta <i>et al.</i> , (2007a)
<i>Proteusmirabilis</i>	Clinical	2	Broth dilution	Hegazy (2016)
	Clinical	1769.5	AWD with MIC*	Mohsen <i>et al.</i> , (2015)
<i>Proteusspp</i>	Clinical	2 – 64 [64]	Agar dilution	Ahmed <i>et al.</i> , (2017)
<i>Salmonella spp.</i>	Clinical	50 - >1000	Agar dilution	Duttaet <i>al.</i> , (2007a)
<i>S. Typhimurium</i>	NCTC 11	100	Agar dilution	Annaduraiet <i>al.</i> , (2002)
<i>S. Typhi</i>	NCTC 74	50	Agar dilution	Annadurai <i>et al.</i> , (2002)
	Clinical	2500	Broth dilution	AL-Janabi et al (2009)
<i>Shigella spp.</i>	Clinical	50 - >100	Agar dilution	Duttaet <i>al.</i> , (2007a)
<i>S. dysenteriae</i>	3 NCTC 102/65	200	Agar dilution	Annaduraiet <i>al.</i> , (2002)
	4a NCTC 515/63	100	Agar dilution	Annadurai <i>et al.</i> , (2002)
<i>Enterobacter cloacae</i>	Clinical	2500	Broth dilution	AL-Janabi <i>et al.</i> , (2009)
<i>E. aerogenes</i>	Clinical	2500	Broth dilution	AL-Janabiet <i>al.</i> , (2009)
<i>Vibriocholerae</i>	Clinical	50 - ≥ 1000	Agar dilution	Duttaet <i>al.</i> , (2007a)
<i>Pseudomonasaeruginosa</i>	ATCC 1045	1024	Agar dilution	Ahmed <i>et al.</i> , (2017)
	Clinical	3125	Agar dilution	Abbas <i>et al.</i> , (2012)
	Clinical	2.000	Broth dilution	Abbas (2015)
	ATCC 1014	1675.7	AWD with MIC*	Mohsen <i>et al.</i> , (2015)
<i>Pseudomonasspp.</i>	Clinical	4 – 256 [256]	Agar dilution	Ahmed <i>et al.</i> , (2017)
		Concentration / Z of Inhibition (mm)		
<i>E. coli</i>	ATCC 9637	2500 / 14	AWD	Umaru <i>et al.</i> , (2009)
	MTCC 443	25-100 / 24-25	AWD	Padma and Yalavarthy (2015)
<i>S. Typhi</i>	ATCC 13709	2500 / 13	AWD	Umaru <i>et al.</i> , (2009)
<i>P. aeruginosa</i>	ATCC 27853	2500 / 8	AWD	Umaru <i>et al.</i> , (2009)
	MTCC 424	25-100 / 24-26	AWD	Padma and Yalavarthy (2015)
<i>S. Typhi</i>	MTCC 98	25-100 / 22-23	AWD	Padma and Yalavarthy (2015)

MIC: Minimum inhibitory concentrations; []: MIC₉₀; AWD: Agar Well Diffusion; *: MIC was calculated by plotting the natural logarithm of the concentration of each dilution against the square of zones of inhibition

Table.2 Diclofenac inhibitory activity against Gram positive and mycobacteria and the methods employed

Bacteria	Source	MIC (µg/mL)	Method	Reference
<i>Staphylococcus aureus</i>	NCTC 6571	50	Agar dilution	Duttaet al., (2007a)
	NCTC 6571	50	Broth dilution	Dastidaret al., (2000)
	Clinical	50 - >1000	Agar dilution	Duttaet al., (2007a)
	ATCC 25923	1250	Broth dilution	Chan et al., (2017)
	ATCC 33591	312	Broth dilution	Chan et al., (2017)
	Clinical	312 – 2500	Broth dilution	Chan et al., (2017)
	ATCC 6538	4.7 (mM)	Broth dilution	El-Baky and El-Gendy (2016)
	Clinical	2.35 – 4.7 (mM)	Broth dilution	El-Baky and El-Gendy (2016)
	Clinical	8 – 1024 [256]	Agar dilution	Ahmed et al., (2017)
	ATCC 6538	1465	AWD*	Mohsen et al., (2015)
	Clinical	615	Broth dilution	AL-Janabi et al., (2009)
	ATCC 25923	[250]	Broth dilution	Alqahtani et al., (2018)
CoNS	Clinical	0.5 – 1024 [256]	Agar dilution	Ahmed et al., (2017)
<i>S. epidermidis</i>	Clinical	250	Broth dilution	Perilli et al., (2000)
<i>Enterococcus faecalis</i>	Clinical	8 – 256 [256]	Agar dilution	Ahmed et al., (2017)
	ATCC 29212	50	Broth dilution	Salem-Milani et al., (2013)
<i>Streptococcus spp.</i>	Clinical	1 – 256 [256]	Agar dilution	Ahmed et al., (2017)
<i>Bacillus spp</i>	Clinical	0.5 – 2 [2]	Agar dilution	Ahmed et al., (2017)
<i>B. pumilus</i>	NCTC 8241	50	Agar dilution	Annadurai et al., (2002)
<i>B. subtilis</i>	Clinical	0.315	Broth dilution	AL-Janabi et al., (2009)
	ATCC 23857	[50]	Broth dilution	Alqahtani et al., (2018)
<i>Listeria monocytogenes</i>	ATCC 51774 and others	[50]	Agar dilution	Duttaet al., 2008a)
	ATCC 51774 and others	[50]	Broth dilution	Duttaet al., (2009)
<i>Mycobacterium tuberculosis</i>	Standard and clinical	10 – 2510	Agar dilution	Dutta et al., (2007b)
<i>Mycobacterium spp.</i>	Clinical	10 - 25	Broth dilution	Dutta et al., (2004)
		Concentration / Z of Inhibition (mm)		
<i>B. subtilis</i>	MTCC 441	25-100 / 18 - 19mm	AWD	Padma and Yalavarthy (2015)
<i>S. aureus</i>	MTCC 96	50-100 / 16 – 21mm	AWD	Padma and Yalavarthy (2015)

MIC: Minimum inhibitory concentrations; []: MIC₉₀; CoNS: Coagulase negative Staphylococci; AWD = Agar Well Diffusion; *: MIC was calculated by plotting the natural logarithm of the concentration of each dilution against the square of zones of inhibition

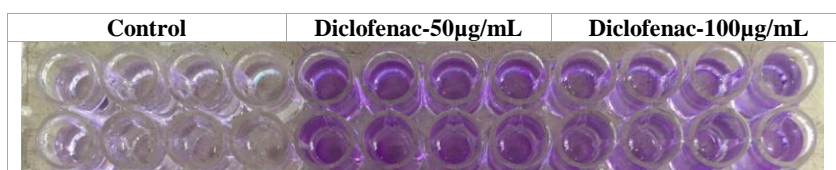


Figure 1. Induction of biofilm by diclofenac in two clinical strains of *S. aureus* (work in progress)

Lawrence *et al.*, (2007) investigated the impact of 10 and 100 µg / L diclofenac on river biofilm communities grown as model systems in rotary annular reactors. However, in contrast to the results obtained by Paje *et al.*, (2002), these authors observed that 100 µg / L of the drug did not reduce bacterial biomass; instead, the direct counts indicated increased bacteria numbers, along with an increase in the thickness of the biofilms. Diclofenac also produced significant effects on the nature of the bacterial community compared to both the control and communities of the river biofilm treated only with nutrients.

Other effects of diclofenac

In addition to affecting biofilm formation, NSAIDs may influence the expression of other factors of bacterial virulence. Subinhibitory concentrations of diclofenac significantly inhibited swarming and swimming motilities of *P. mirabilis* isolated from a diabetic foot, indicating that the spread of infection could be affected by this NSAID. Noticeable decreases in or inhibition of protease, hemolytic and urease activities were also observed (Hegazy, 2016).

In a similar study with *Pseudomonas*, Abbas (2015) showed that subinhibitory concentrations of diclofenac equally decreased twitching and swimming motility, affected protease and hemolytic activities, and induced a significant decrease in the production of pyocyanin and ploverdine. Decreases in the production of regulated quorum sensing virulence factors (elastase and pyocyanin) and in swarming motility were also previously reported as effects of diclofenac in *P. aeruginosa* (Ulusoy and Bosgelmez-Tinaz, 2013).

It is concluded that, due to the rapid increase and global spread of antimicrobial resistance

over the last decades, there are currently few effective conventional therapeutic options for the treatment of certain infections involving multiresistant pathogens. The problem is aggravated in biofilm-associated infections, which, when present, create a barrier that prevents antimicrobial access.

The pharmaceutical industry's difficulties in responding to antimicrobial resistance have made the establishment of alternative approaches to the control of such infections even more urgent. In this sense, the use of "non-antibiotic" drugs represents an interesting approach, either for single use or as adjuvant to conventional antibiotics. However, some limitations emerging from the studies on diclofenac must be taken into account.

The MICs reported in the different investigations for this drug show great variation, which may be the result of methodological factors, such as the type of solvent used in the dissolution of the drug (for example, distilled water instead of methanol or DMSO), culture media and techniques used by the authors.

Further clinical studies are required, as well as a better understanding of the effect of subinhibitory concentrations of this NSAID on bacterial biofilm formation.

An obstacle to the current use of diclofenac as an alternative to the conventional antibiotics, or as an adjuvant drug to the antibiotics, is that, with a few exceptions, the MICs obtained in in vitro tests are above those achieved in serum. However, the fact that diclofenac penetrates efficiently into inflamed tissues and synovial fluid – where high concentrations are maintained compared to plasma concentrations – should be taken into account. High concentrations of the drug can also be achieved with topical therapy.

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