Benzotriazole: an overview on its versatile biological behaviour

BENZOTRIAZOLE: AN OVERVIEW ON HIS VERSATILE BIOLOGICAL BEHAVIOURS

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ABSTRACT

Discovered in late 1960, azoles are a class of heterocyclic compounds that constitute the largest group of available antifungal drugs, and the imidazole ring is the synthetic component that confers activity to azoles. Triazoles are obtained by a slight modification of this ring and present similar or improved activity and less adverse effects. Consequently it is not too surprising that benzimidazole/benzotriazole containing molecules and benzimidazole/benzotriazole derivatives have been found to be biologically active. Since benzimidazole has been widely investigated, this review is focalized on define the place in biomedical research of benzotriazole derivatives, highlighting their versatile biological properties, the mode of action and Structure Activity Relationship (SAR) studies for a variety of antimicrobial, antiparasitic, and even antitumor, choleretic, cholesterol-lowering agents.

Keywords: 1H-benzo[d][1,2,3]triazole; antimicrobial; antiprotozoal; antiviral; antimycobacterial; anti-tubulin.

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INTRODUCTION

1*H* -benzo[*d*][1,2,3]triazole (Fig. 1), in this context more simply called benzotriazole (BT), may be considered for its several pharmacological activities as privileged structures, and potentially useful as scaffold for the design of new pharmacologically active compounds, undergoing rapid development in the synthesis of heterocycles.

Fig. 1. Chemical structure of 1*H*-benzo[*d*][1,2,3]triazole.

From a purely chemical point of view, the benzotriazole structure proved extremely versatile applicability, for instance being used as a intermediate [1-5] or as a good leaving group after reaction with a variety of carbonyl groups [6-9]. An example of his use as a reaction intermediate is given by the use that some researchers have done of the acylbenzotriazole methodology, taken from Katrizsky and collaborators. [10]. The *N*-acylbenzotriazole is an easy-to-handle acylating agent for advantageous N-, O-, C- and S-acylations, and dicarboxylic benzotriazole derivatives were used to obtain new peptidomimetic macrocycles, (Fig. 2), that showed antibacterial properties [11].

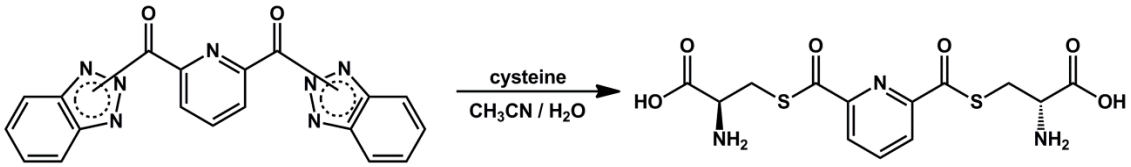


Fig. 2. BT as leaving group.

The benzotriazole, in fact, can be considered a good leaving group, but also acts as an electron-donor and a precursor of radicals or carbanions. It is easily insertable into other chemical structures through a series of reaction, such as condensation, addition reactions and benzotriazolyl-alkylation [12-14]. Some authors have also reported the synthesis of stable nitrenium ions using benzotriazole as synthon [15].

However, the main interest on BT is focused in the pharmaceutical field, as suitably substituted benzotriazole derivatives can boast the most diverse pharmaceutical properties, including plant-growth regulator [16-19], choleretic [20], antibacterial [21], antiprotozoaric [22], antiviral [23] and antiproliferative [24] activities.

BENZOTRIAZOLE AS ANTIMICROBIAL AND ANTIPROTOZOAL AGENT

Antimicrobial activity of benzotriazole derivatives has been extensively investigated since the late 1980s, and together with all azolic rings they have become one of the active highlights in recent years [25]. In the first part of the twentieth century the discovery and development of antibacterial drugs were major scientific achievements, even if no new drug class has been discovered in the past 20 years despite the investments in antimicrobial drug discovery [26]. Furthermore, antibiotics *resistance* also make infections more difficult to treat, highlighting the urgent need for new drugs [27]. For some decades Sparatore and co-workers studied and characterized various nitrogenous rings and reported that when benzotriazole is part of larger heterocyclic system bears biological activities, antibacterial in particular) [28-30]. In 1989 Sanna's research group also reported the relevance of the benzotriazole moiety in triazolo[4,5-*f*]-quinolinone carboxylic acids, closely related to oxolinic acid, showed encouraging *in vitro* antimicrobial activity against *E. coli*, with MIC values

included between 12.5 and 25 μg/ml. In fact a different annulations position of the triazole ring as in triazolo[4,5-*h*]-quinolinone carboxylic acids is not profitable, causing a partial or total antimicrobial activity loss [31, 32]. More compounds are reported by Al-Omran and co-workers, which synthesized a variety of benzotriazole derivatives incorporating thiophene, pyridine, thiadiazole or pyrazole moiety: screened for antimicrobial activity, most of tested samples resulted bactericidal [33].

Purohit and Srivatava synthesized and screened a series of chlorosubstituted, phenoxyacetyl and propionyl benzotriazoles: all compounds showed mild to moderate antibacterial and antifungal activity, thus demonstrating that even the simple benzotriazole derivatives posses antibacterial properties [34]. Similar activity is reported for a series of 1-(*N*-heteroyl/diphenyl amino acetyl/propionyl) benzotriazoles [35]. 1*H*-Benzotriazol-1-yl(2-hydroxy-5-[(*E*)phenyldiazenyl]phenyl)methanone derivatives, prepared by Jamkhandi and co-workers through diazonium coupling reaction, showed good antibacterial and antifungal activities, with remarkable zone of inhibition in comparison with standards drugs [36]. Same results were also reported for [(1*H*-benzotriazol-1-ylacetyl)amino]acetic acid derivatives [37], while 2-(1*H*-1,2,3-Benzotriazol-1-yl)-*N*-phenylacetamide derivatives displayed less antibacterial potency [38].

By Ochal group is also reported the *in vitro* antibacterial activity of 5-halogenomethylsulfonyl-benzotriazoles, all tested against a series of reference (including Gram-positive and Gram-negative bacteria) and clinical strains (including methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) *S. aureus* strains, plus methicillin-resistant *S. epidermidis*). Compound with trifluoromethyl- substituent at C-2 position displayed significant antibacterial activities comparable with nitrofurantoin against some strains, being able to inhibit *Staphylococci* strains (MRSA) with MIC values 12.5-25 μg/mL [39].

Benzotriazole can also be used to modulate the biological activity of other heterocyclic nuclei, as for 9-substituted acridines reported by Singh *et al.* [40], or for levofloxacin derivatives, where 2-aminobenzotriazole is linked to the carboxyl moiety. In this second case, the modification led to a comparable antimicrobial activity against Gram-positive and Gram-negative bacteria in comparison with the levofloxacin nucleus [41]. Das *et al.* synthesized and evaluated as antimicrobial agents some novel oxazolidinone derivatives with benzotriazole as pendant, which relevance was confirmed after his replacement with benzimidazole, benzthiazole, or benzoxazole, modifications that provide less active or inactive the molecules. Effects of positional and geometrical isomerism on triazole moiety demonstrated that linearly attached benzotriazole derivatives showed more potency compared to angular ones *in vitro*, while, for angularly attached derivatives, *E*-isomer was found to be more potent than *Z*-isomer. Finally, thioacetamide analogue of linear compound gave a

lead compound possessing similar activity to linezolid *in vitro* [42]. A series of novel substituted and unsubstituted benzotriazolyl oxazolidinone derivatives was also evaluated against a panel of susceptible and resistant Gram-positive and Gram-negative bacteria, some of which resistant to methicillin and vancomycin, and some of them were found to be *in vitro* equipotent or more potent than linezolid [43]

Asati's group linked benzotriazole nucleus to 4-oxo-thiazolidines and their 5-arylidene derivatives, obtaining 5-arylidene-2-aryl-3-(benzotriazoloacetamidyl)-1,3-thiazolidin-4-ones. The derivatives were screened against *Bacillus substilis*, *Salmonella typhimurium*, *Escherichia coli* and B*acillus anthracis* at 50 and 100 ppm, and some of them resulted equipotent to streptomycin [44].

In 2006 Swamy *et al.* prepared a series of *N*-alkylated benzotriazole derivatives through a microwave-assisted synthesis [45], as reported in Fig. 3.

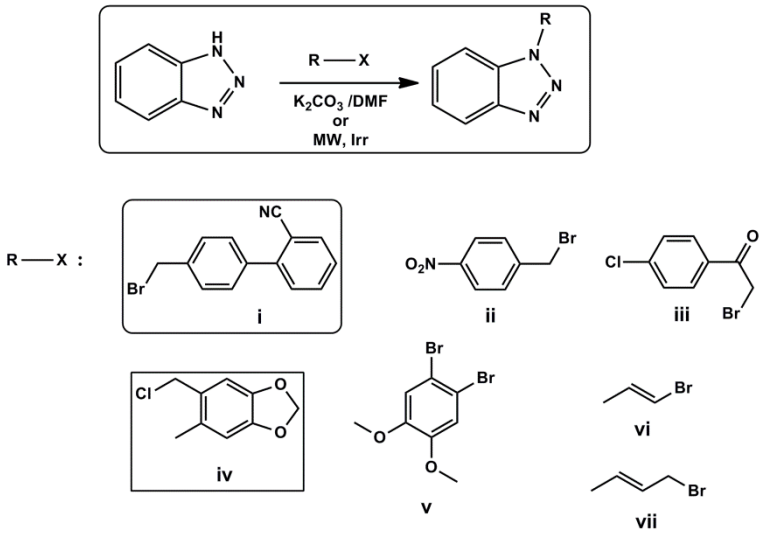


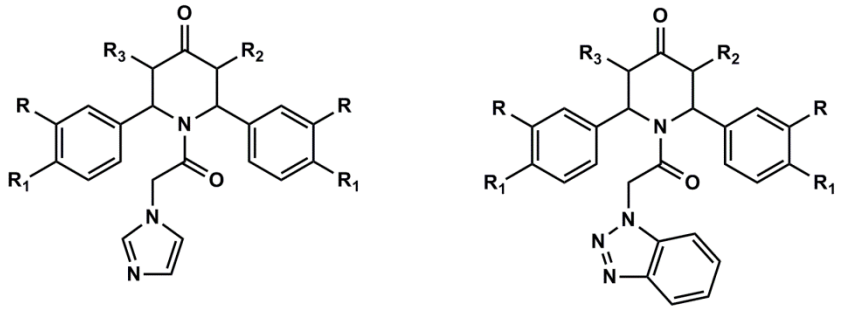
Fig. 3. Microwave-assisted synthesis of N-substituted benzotriazole derivatives.

The antibacterial activity of all compounds was tested against bacterial strains like *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*, *Xanthomonas campestris* e *Xanthomonas orza*. Results highlight how 4'-((1*H*-benzo[*d*][1,2,3]triazol-1-yl)methyl)-[1,1'-biphenyl]-2-carbonitrile (**i**) and 1-(6-methylbenzo[*d*] [1,3]dioxol-5-yl)methyl)-1*H*-benzo[*d*][1,2,3]triazole (**iv**) act as potent antimicrobial agents, behavior probably due to the presence of bulky hydrophobic groups (cyano-biphenyl and benzodioxole), while smaller derivatives bearing 5-dimethoxy-benzyl (**v**) and 1-butyl (**vii**) groups outcome equipotent to Streptomycin, used as reference drugs.

Finally, even if some derivatives demonstrate to be active on drug-resistant bacterial strains, all of them lack of selectivity, being active on both Gram-positive and Gram-negative bacteria, over on different fungal strains.

Papers by different authors report that imidazole and triazole nuclei are isosteric analogs,

synthetically relevant, associated to a variety of biological and pharmacologic activities [46]. In the same way the N substitution for CH, that transform benzimidazole in benzotriazole derivative, can be considered an example of a classical isosteric replacement [47]. Leading this idea, Ramachandran and collegues synthesized and evaluated the antibacterial and antifungal activities against various pathogenic microbial strains of a series of new imidazole/benzotriazole substituted piperidin-4-one derivatives, as reported in Fig. 4 [48].



1-6 **7-10**

Comp.	R	R_1	R_2	R_3
1	H	H	CH_3	H
2	H	H	CH_2CH_3	H
3	H	H	$CH(CH_3)_2$	H
4	H	H	CH_3	CH_3
5	F	H	CH_3	CH_3
6	H	OCH_3	CH_3	CH_3
7	F	H	CH_3	CH_3
8	H	F	CH_3	CH_3
9	H	OCH_3	CH_3	CH_3
10	H	F	CH_2CH_3	H

Fig. 4. Imidazole/benzotriazole substituted piperidin-4-one derivatives.

This study highlights how the presence of bulky substituents, such as the isopropyl group, at position C-3 of piperidinic cycle increases the antibacterial activity against *B. subtilis* for both imidazole (**3**) and benzotriazole (**10**) derivatives (MIC = 12.5 and 6.25 µg/ml, respectively), in comparison to reference drug Streptomycin. A similar behavior is not observed in antifungal test. Additionally, a methyl group at C-3 and C-5 along with *m*-fluorophenyl (**5**) and *p*-methoxyphenyl (**8**) at C-2 and C-6 exerted good growth inhibition toward *E. coli* (MIC = 6.25 µg/ml and 12.5 µg/ml, respectively). Surprisingly, the introduction of an isobutane group in C-3 and with a CH in 5 leads to a higher inhibitory activity against *B. subtilis* (MIC = 6.25 µg/ml) [39]. Most of the compounds demonstrate nonexistent or moderate growth inhibition activity on different fungal strains, exception for compounds **3** and **8** against *A. niger* (12.5 µg/mL), **5** against *Candida neoformans* (12.5 µg/mL), **6** against *Candida albicans* (6.25 µg/mL) and **8** against *Rhizopus sp.* (6.25 µg/mL).

Finally, Suma's research group investigated the benzotriazole antimicrobial proprieties introducing not only electron withdrawing substituent as Cl or NO_2 in the phenilyc moiety, but also variously

substituted pyrazilidin-3,5dione moieties linked to the heterocyclic nucleus [49]. All synthesized compounds were characterized and biologically evaluated by cup plate diffusion method. Between the examined derivatives, showed in Fig. 5, only compound **viii** resulted comparable to Ciprofloxacin on *Staphylococcus. Aureus*, while a limited activity is reported toward *C. Albicans*. Unfortunately, no SAR analysis is reported.

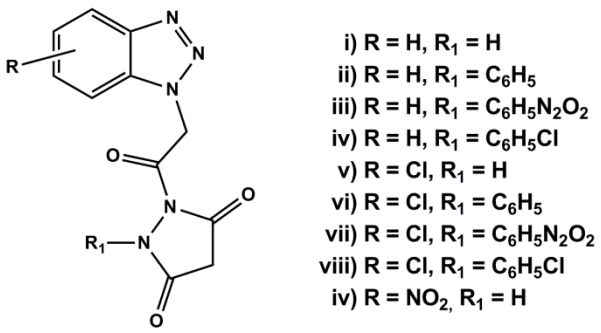


Fig. 5. 1-(2-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)acetyl)2-R_1-pyrazolidine-3,5-dione derivatives.

Finally, little is known about the antiprotozoaric activity of benzotriazole derivatives. A simply substitution on BT generate 6-chloro-1*H*-benzotriazole, a compound endowed with micromolar activity against *Entamoeba histolytica*, demonstrating that this scaffold, as indazole nucleus, is more active than metronidazole, the drug most commonly used in the treatment of anaerobic protozoan parasitic infections [22]. Pagliero's works demonstrated that compounds possessing the heterocyclic nucleus 2-methyl-1,2,3,4-tetrahydroquinoline linked to a benzenesulfonyle moiety showed protozoan antiparasitic proprieties [50, 51]. On this basis, through the fragment-based drug design strategy, Becerra's group designed and prepared a library of *N*-benzenesulfonyl derivatives of BT, replacing the H at N_1 with substituted-benzenesulfonyl groups, all compounds prepared by sulfonylation, as reported by Katrinsky [52]. Researcher determined *in vitro* the biological activity of these compounds on the protozoan parasite *Trypanosoma cruzi*. Adding different concentrations of *N*-benzenesulfonylbenzotriazole to the growth medium on epimastigote and trypomastigote forms, they demonstrate that derivatives have an *in vitro* growth inhibitory dose-dependent activity against epimastigotes. Particularly, after incubation for 72 h the parasite number in epimastigotes form decrease of about 50% at 25 μg/mL and of 64% at 50 μg/mL. In the same conditions BT, used as reference compound, did not show anti-trypanosomal activity. On trypomastigotes, the infective form of the parasite, *N*-benzenesulfonylbenzotriazole appeared to be even more effectives: at 50 μg/mL it was effective at an earlier time than on epimastigote forms, causing a percentages of trypomastigotes dead higher than 95%. BT was less effective, causing at the same concentration only 21% of dead parasites [53].

BENZOTRIAZOLE AS ANTIMICOTIC AGENT

As previously reported, several authors reported the biological evaluation of imidazole derivatives and benzotriazole analogues as antibacterial and antimicotic agents, and for these derivatives, a selective action is not demonstrated [45, 48]. However, the structural model of the best-known antifungal drug, fluconazole, offers an interesting starting point for drug design studies on the triazolic system, which can then be replaced by a benzotriazole nucleus, in order to evaluate the effect of the bioisosteric replacement on the biological activity. Concerning the mechanism of action, is known that antimycotic drugs, such as fluconazole [54], itraconazole [55], voriconazole [56], ravuconazole [57] and posaconazole [58] exert their pharmacological action by inhibiting the fungal 14 α-demethylase cytochrome P450, known as CYP51, an essential enzyme in the biosynthesis of sterols. In particular the CYP51 removes by oxidation the 14 α-methyl group of lanosterol, using oxygen and NADPH, transforming it into ergosterol, an essential component of the fungal cell membrane [59]. These drugs act by displacing lanosterol from CYP51 binding site, causing a block in the biosynthesis of ergosterol and an accumulation of 14 α-methylsterols [60].

For fluconazole and analogues, crucial interactions at the enzymatic active site are favored by these components: 1) the basic nitrogen atom in position 3 in the triazole moiety, forms a bond with the acid iron of the CYP51 heme prosthetic group, in a position normally occupied by the oxygen, 2) the presence of aromatic rings and 3) the molecular behavior almost non-polar [61], as described in Fig. 6.

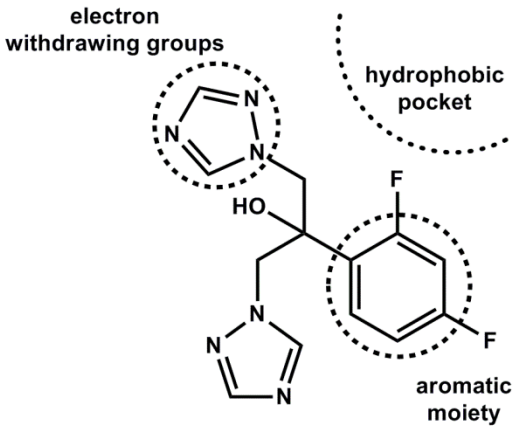


Fig. 6. Schematic view of the key interaction between fluconazole and its receptor.

Now, fluconazole is effective against candidiasis after both *oral* and parenteral administration, but is ineffective against aspergillosis. An increasing incidence of infections caused by *Candida not albicans*, such as *C. glabrata* and *C. krusei* resistant to this drug was also observed [62]. These evidences have greatly stimulated the pharmaceutical research in the antimycotic field, leading to the synthesis of new derivatives having as central structure a benzotriazole group. These

compounds, in comparison with imidazole antifungal drugs, seems to possess a broader antifungal spectrum paired with a lower toxicity [63]. From this perspective, Patel's research group modified the 1,2,4-triazole ring of fluconazole to 5(6)-(un)substituted benzotriazole lead compounds (Fig. 7) [64].

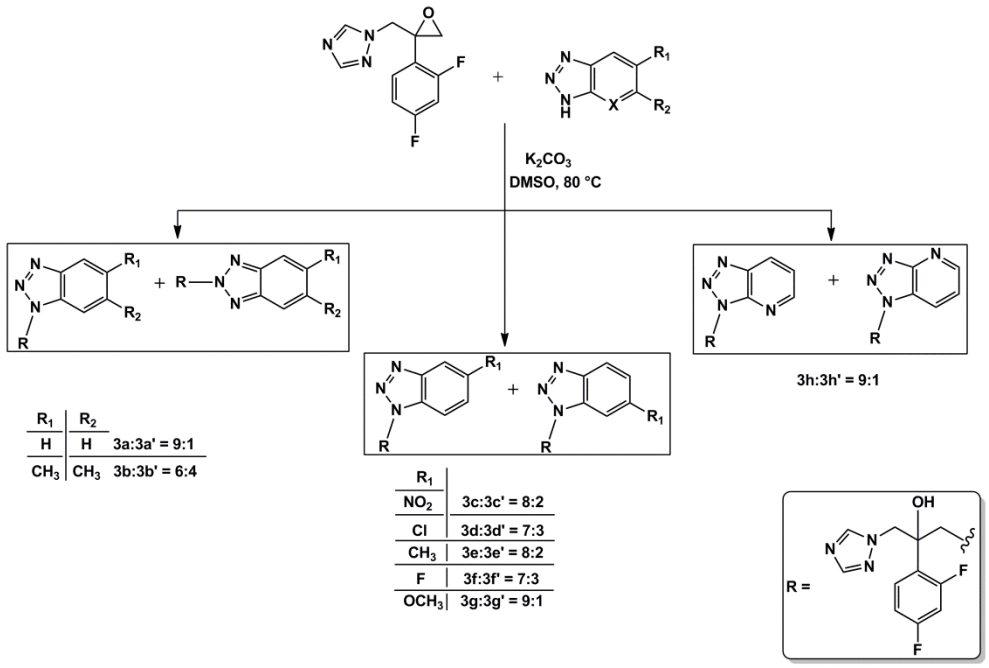


Fig. 7. Synthesis scheme of 5,6 substituted benzotriazoles derivatives endowed with antifungal activity.

Particularly, 5,6-dimethylbenzotriazol-2-yl, 5-chlorobenzotriazol-1-yl and 6-methylbenzotriazol-1-yl derivatives exhibited potent antifungal activity, with MICs values on *Candida spp.* ranging from 1.6 μg/ml to 25 μg/ml. Finally, the introduction on the benzotriazole ring of the small hydrophobic groups –Cl, $–CH_3$ and di–CH_3, lead to compounds successful against both *Candida* and A*spergillus spp.*, with 12.5-25 μg/ml MIC values on *A. niger*. Replacement of the benzotriazole ring with a triazolopyridinic nucleus generate a polar analogue less potent. Finally, the replacement of hydrogen in position 5 of the benzotriazole with electron-withdrawing groups such as -NO_2, -Cl, -F, increases the antimycotic activity; on the contrary, the same substitution at position 6 of the ring leads to compounds definitely less potent.

XP-Glide docking method analysis demonstrated the relevance of hydrophobic substituents in the molecular interaction with the *Mycobacterium tuberculosis* CYP51 (MT-CYP51), selected model in the absence of crystal structure of fungal CYP51. Particularly, for S enantiomers a lower glide score (expressed in Kcal/mol) is reported; indeed, they have an antimycotic activity higher than the respective enantiomers R. Furthermore, to evaluate interaction differences at the binding site, CYP51 aminoacid sequences of *Mycobacterium Tuberculosis* and *Aspergillus niger* have been compared. The obtained results showed that antifungal potency differences among benzotriazole

regioisomer couples depended on the interactions of substituents with the aminoacids contained by MT-CYP51 active site, but it is mainly due to the triazole heterocycles placement into it.

Also Rezaei and colleagues synthesized a set of triazolic derivatives, which included benzotriazole compounds targeting the CYP51. Obtained compounds, reported in Fig. 8, had a molecular weight between 139 and 421 kDa and, as fluconazole, the molecular docking simulation was performed at the level of the CYP51 active site.

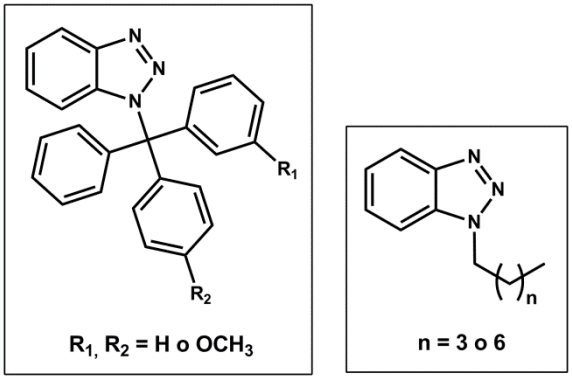


Fig. 8. Chemical structure of the 1,2,3-benzotriazole derivatives proposed by Rezaei *et al.*

Collected data showed values such as these obtained for fluconazole; in particular, lower FDE values, index of better drug-target interaction, were obtained with 1,2,4 triazole derivatives. However, there is no correlation between antifungal activity and FDE. These analysis underlined that the antimicrobial potency was related to the molecular penetration into fungi cell, and the presence of bulky substituents, as OCH_3, is believed to be responsible for the decrease of this activity [65, 66].

In following research, the team headed by Rezaei continued the investigation on CYP51 inhibitors synthesizing new 1,2,4-triazole, imidazole, benzimidazole and, interesting for us, benzotriazole derivatives (Fig. 9). Throughout drug-design studies, applying Autodock on MT-CYP51, the best *in silico* promising derivative were synthesized and their antimicotic activity was compared to that of fluconazole and itraconazole, used as reference drugs [66].

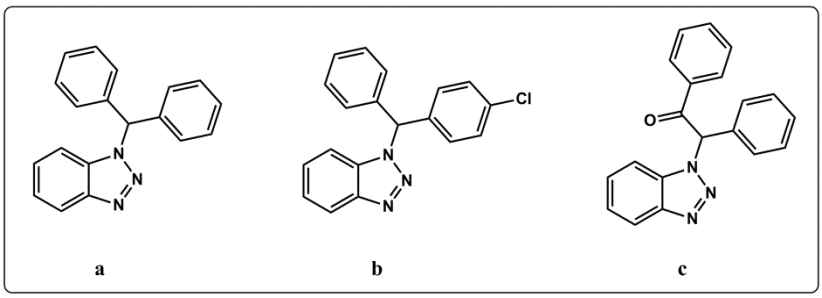


Fig. 9. Biphenyl and benzhydryl benzotriazole derivatives.

Unfortunately, obtained results from this studies showed that benzotriazole derivatives do not have antifungal activity against different *Candida* species. Exceptionally, these compounds possess

activity against the dermatophyte *Microsporum canis*. The only compounds active against all microbe species taken into account are derivatives of 1,2-diphenyl-2-(1*H*-1,2,4-triazol-1-yl)ethanone, 1-(diphenylethil)-1*H*-imidazole and 1-[(4-chlorophenyl)((phenyl)methyl]-1*H*-imidazole. This activity was probably due to their lower size, which allow a better penetration into the fungi cell, and the presence of the five-membered nitrogen heterocyclic ring. Finally, in the benzimidazole series, inserting two substituents $–OCH_3$ on phenyl *para* position induce total loss of antifungal activity; whereas, inserting an ethyl-piperazine reduce antifungal potency in the imidazole series.

Concerning the same azole antifungal model, a novel work was presented by Gaikwad and colleagues [67], wherein the benzotriazole core was modified by heterocyclic alkylation with complicated side chains bearing a substituted thiazolic ring (Fig. 10).

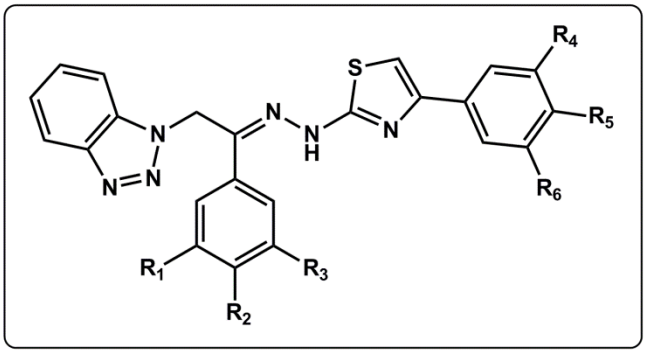


Fig. 10. Benzotriazol-1-yl-1-(phenyl)ethylidene)hydrazinyl)-4-phenylthiazole derivatives chemical structure.

Tested on a wide number of microorganisms, Gram-positive, Gram-negative and fungi, in both cases the best activities were observed for derivatives bearing electron withdrawing substituents as F, Cl, Br, and NO_2 on side phenyl groups, independently by their position on the ring. More precisely, these derivatives were able to inhibit the growing of Gram-positive and Gram-negative bacteria in equal way, as well as *C. Albicans* and *A. Niger* in a comparable way as Nistatine.

BENZOTRIAZOLE AS ANTIMYCOBACTERIAL AGENT

Despite the perception, nowadays Tuberculosis (TB) is still one of the most common infectious diseases, resulting in as many as one-third of world's population (~2 billion people) infected with *Mycobacterium tuberculosis* (*M. tb*) [68]. Every year about nine million new cases of tuberculosis occur and up to two million individuals die due to this disease [69]. Even more frightening is the rapid emergence of Multi-Drug-Resistant (MDR) [70] and Extensively-Drug-Resistant (XDR) strains [71] in all regions of the world, along with the dangerous synergy between the Human

Immunodeficiency Virus (HIV) and *M. tb* [72, 73]. TB remain a leading cause of death among HIV positive patients [74].

The treatment of active tuberculosis require a combination of several drugs, such as isoniazid, pyrazinamide, ethambutol, rifampicin and streptomycin [75], all used in various combinations as first-line therapy [76]. On the contrary quinolones are classified as second-line drugs [77], even if their use is recommended in managing MDR-TB [78]. However, due to outcoming of MDR and XDR TB strains, worldwide there is an urgent need for new drugs having a different mechanism of action from those mentioned above, also to short the duration of pharmaceutical therapy.

Various research groups focalized their attention on this field and a large amount of molecules have been tested for this purpose [79-82]. Since ‘90s Carta and co-workers focalized their attention on the bioisosteric modification of these heterocycles creating various benzotriazole derivatives. A first series of 3-aryl substituted-2-(1*H*(2*H*)benzotriazol-1(2)-yl)acrylonitriles [83] was prepared with the aim to characterize the best substituents connected with the aryl moiety selecting R within groups holding various electron-accepting or donor properties and lipophilic–hydrophilic balance. During the synthesis, the performed Knoevenagel condensation had as result in most cases the only *E*-isomer formation, while in a few cases a mixture of *E/Z*-isomers was observed. From this preliminary study 1-substituted benzotriazole derivatives resulted more active than the 2-benzotriazolyl isomers, while the unsubstituted phenyl moiety (**d**) outcome the best option, exhibiting the highest antimycobacterial activity *in vitro*, also against *Mycobacterium avium*. The only exception is represented by 4-bromophenyl derivative (**e**), even if the activity resulted lower than that of the unsubstituted terms, as depicted in Fig. 11.

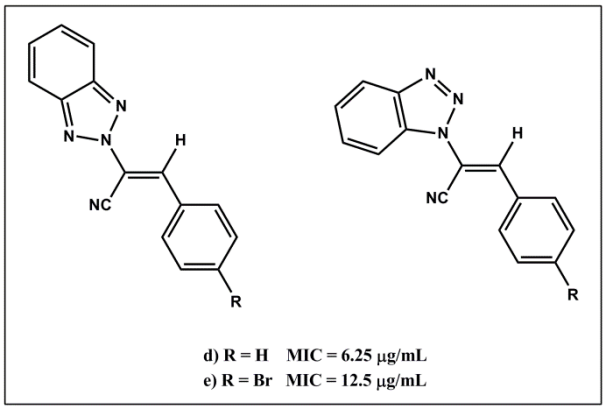


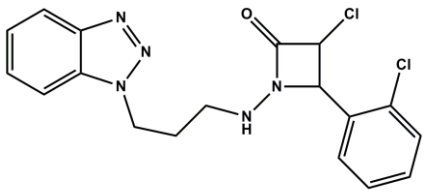
Fig. 11. 3-aryl substituted-2-[1*H*(2*H*)benzotriazol-1(2)-yl]acrylonitriles general formula and MIC values of the compounds **d** and **e**.

With the aim to improve the antimycobacterial activity, further modifications, such as the introduction in the phenyl ring C-4 of two or more electron-releasing groups, or the replacement of the phenyl ring with cyclohexyl or larger aromatic rings, were performed [84]. Unfortunately the

produced 3-aryl-, 3-cyclohexyl and 3-heteroaryl substituted-2-(1*H*(2*H*)-benzotriazol-1(2)-yl)prop-2-enenitriles, prop-2-enamides and propenoic acids showed a strong reduction or a loss of the activity in spite of their increased lipophilic character. This indicate that the steric hindrance, as well as the substituents nature, may play a relevant role in determining the ability to inhibit the *M. tb* proliferation. Finally, in order to extend the SAR studies, various substituents were introduced alternatively at position 2′, 3′ and 4′ in the phenyl moiety [85]. Both the previously synthesized and the new derivatives were evaluated for cytotoxicity against MT-4 cells. Unluckily, the relevant cytotoxicity showed by many derivatives induced to dispose of acrylonitriles as antitubercular and to evaluate them as antiproliferative agents, as above reported.

Nevertheless, benzotriazole remain an interesting heterocycles for his antimycobacterial proprieties, and various groups tried to combine chemical structures of well know antibacterial agents within benzotriazole, designing and synthesizing new molecular series with the aim to develop new antitubercular agents able to succeed toward *M. tb* sensitive and resistant strains.

Dubey *et al.* coupled the benzotriazole proprieties with the antimicrobial activities of the *β*-lactams 2-azetidinones [86, 87]. The 2-oxo-4-substituted aryl- azetidinone derivatives of benzotriazole were prepared by both conventional and microwave irradiation, this one outcoming the best performing synthetic way. Resulting no cytotoxicity for all of the prepared compounds, the derivatives were tested against *M. tb* and some other microorganisms, such bacteria and fungi. The best product of this series was compound **f**, endowed with a promising activity against *M. tb* (MIC = 3.125 μg/ml), but also active toward some bacteria (MIC = 0.1 μg/ml on *Escherichia Coli*) and fungi (MIC = 0.5 μg/ml on *Aspergillus niger),*(Fig. 12). For this class of compounds no selectivity of action is reported.



f

$MIC_{(on\ M.Tb.)}$ = 3.125 μg/ml

Fig. 12. 2-oxo-4-substituted aryl-azetidinone derivatives of benzotriazole.

Dixit *et al.* used as basis scaffold the Linezolid structure to create a new class of oxazolidinones derivatives [88] (Fig. 13), performing a bioisosteric substitution of the morpholinic moiety with the benzotriazole nucleus.

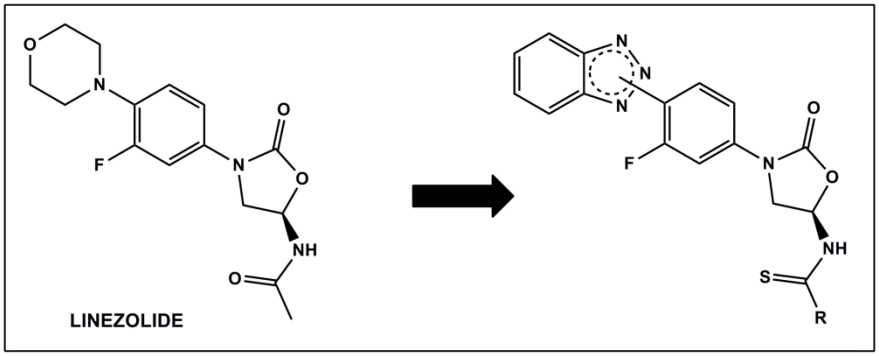


Fig. 13. Bioisosteric substitution on the structure of Linezolide.

Nine 1-[3-(4-benzotriazol-1/2-yl-3-fluoro-phenyl)-2-oxo-oxazolidin-5-ylmethyl]-3-substituted-thiourea derivatives were synthesized starting from commercial benzotriazole and the antimycobacterial activity was determined against susceptible (sensitive strains; inhibited by the two front line anti-TB drugs such as isoniazid or rifampicin) and resistant strains (not inhibited by either isoniazid or rifampicin or both). The (R)-N-(3-(4-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)-3-fluorophenyl)-2-oxooxazolidin-5-yl)cyclopropanecarbothioamide sort out to be the best derivative, endowed with an excellent in vitro profile against *M. Tb.* H37Rv (MIC = 0.06 μM) and both drug sensitive and resistant clinical isolates (MIC = 0.125–1.0 and 1–2 μM respectively). These MIC values were equivalent to linezolid and superior to isoniazid against all strains.

As observed until now, the applied strategies to design a new antitubercular agent normally scheduled the bioisosteric modification of a know structure to obtain new linear derivatives. In this perspective an exception is represented by a series of angular derivatives synthesized by Carta *et al.*, structurally related to quinolones, one of the most widely prescribed family of synthetic antimicrobial agents. In particular Fluoroquinolones, fluorine-containing derivatives of older quinolones such as nalidixic acid, are currently used as antitubercular agents in MDR-TB infection and, to a lesser extent, in the case of severe adverse reactions to the conventional antituberculous regimen [89]. However, they are still classified as second-line drugs, since their use in tuberculosis treatment remains controversial [77]. This behavior is justified by the fact that the emergence of fluoroquinolone resistance in MDR strains is possible [90], even if it does not appear to be related to poor bactericidal activity but to rapid emergence of resistance at the doses used clinically, as seen for ciprofloxacin [91].

Quinolones mechanism of action and pharmacokinetics are widely studied, and various review can be found in literature [92-95]. Aiming to exploit the pharmaceutical peculiarities of this antibiotic class, Carta *et al.* created a new series of [1,2,3]Triazolo[4,5-*h*] and [4,5-*f*]quinolones with the purpose to obtain new more potent and selective agents toward *M.tb* sensitive and resistant strains

[32, 96, 97]. As reported by Milata review [98], the Carta's group discovered that only few triazolo[4,5-*h*]quinolone carboxylic acids exhibited interesting low MIC_{90} values (5.0-1.6 μg/mL), observing also that the activity was related to the length and position of the substituent at triazolo-nitrogen. A methyl in N-3 position has showed the higher activity. This first observation prompted the group to focalize the study designing and selectively synthesizing a series of 3-methyl-9-substituted-6-oxo-6,9-dihydro-3*H*-[1,2,3]-triazolo[4,5-h]quinolone-carboxylic acids, where a variety of substituents on the quinolone nitrogen were introduced to improve activity. All derivatives were tested against *M. tb* H37Rv and further 11 clinically isolated strains endowed with different drug resistance. 3,9-dimethyl-6-oxo-6,9-dihydro-3*H*-[1,2,3]triazolo[4,5-*h*]quinoline-7-carboxylic acid stood out as the most potent derivative, exhibiting MIC_{90} = 0.5 μg/mL against all tested strains paired with no cytotoxicity.

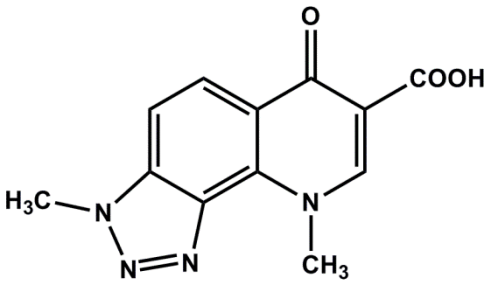


Fig.14. 3,9-dimethyl-6-oxo-6,9-dihydro-3*H*-[1,2,3]triazolo[4,5-*h*]quinoline-7-carboxylic acid.

The latter derivative was selected as lead compound for further examination. Human macrophages J774-A1 were infected with *M. tb* H37Rv strain and successively grown in the absence of antitubercular agent or in the presence of the lead compound at both the concentrations of 0.5 and 0.25 μg/mL. After 7 days the macrophages were lysed and the growth of mycobacterial culture was of 5000 and 8000 CFU/mL, respectively, while the untreated culture grew regularly. In addition, from supplementary analysis a good antimycobacterial activity emerged against several *M. avis paratuberculosis*, *M. smegmatis* and *M. bovis* strains [99]. Further modifications of the substituents in N-3 and N-9 positions of the ring system with bulking groups do not improved the reseached activity, and comparing the new derivatives with the previous series, methyl group was confirmed as the most effective substituent at both positions [100]. Lastly, when MICs were determined against a panel of Gram-positive and Gram-negative bacteria and against *Candida sp.*, all compounds resulted inactive (MIC = 64-100 μg/mL). These actions suggest for this new class of quinolones a specific antimycobacterial potential due probably to a different mechanism of action toward quinolones, leading them to be good candidates for further developments [101].

BENZOTRIAZOLE AS POTASSIUM CHANNEL ACTIVATOR

The activators of the potassium channels represent an emerging class of drugs for the treatment of disorders of the nervous, respiratory and cardiovascular systems, and for this reason represent an interesting research field [102]. From literature, (1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl- 2(3*H*)benzimidazolone), or NS1619, pop up as an activator of the calcium-dependent potassium channels. This molecule is able to determine *in vitro* not only cellular hyperpolarization through direct activation of BKCa, but also a Ca^{2+} channel block: both of these effects contribute to its vasorelaxant properties [103].

Based on NS1619 properties, the Baragatti and Biagi research group conducted broad studies that primarily leaded to the design and synthesis of a series of new derivatives, including 5- substituted-carboxamido-triazolyl-benzotriazoles (proposed as bioisosteric substitution of benzimidazole with benzotriazole moiety), and the corresponding series of 5-substituted-carboxamido-triazolyl-benzimidazolones (Fig. 15, series I).

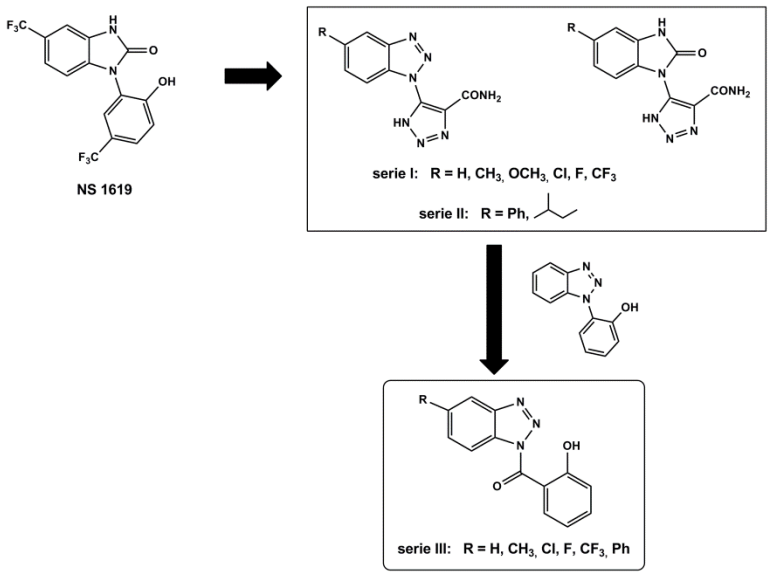


Fig. 15. Potential activators of potassium channel opener.

For all benzotriazole derivatives are reported vasorelaxing properties and potency parameters a lower than that of the reference compound NS 1619, being able to completely break down the contractile tone induced by administration of KCl 20 mM on rat aorta (efficacy 100%). In contrast, benzimidazolone compounds were found to be ineffective or inactive. Particularly, SAR studies shown that the absence of substituents at position 5 on the benzotriazole moiety leads to decrease in potency but not in efficacy, while only the presence of a methoxy group adversely affects both properties. The best results have being observed when the substituent is simple a methyl group. Experimental data seem to suggest therefore a possible correlation between the potency and the steric hindrance in this position. Finally, the benzotriazole derivatives lacking the carboxamido group in position 4 of the triazole ring are totally vasorelaxing powerless, and this demonstrate that the $CONH_2$ group represents an essential structural requirement to exert the vasorelaxing effect due

to the potassium channels BK activation [104].

For this reason, the analisys was carried out studying the effects of steric hindrance and lipophilicity caused by the substituent at position 5. The introduction of bulky and lipophilic groups, such as sec-butyl and phenyl, leads to derivatives retaining equivalent power, but not greater than derivatives bearing a methyl group in the same position (Fig. 15, series II). Alongside these derivatives was then prepared the 1-(2-hydroxyphenyl)-benzotriazole. This derivative showed good vasorelaxing activity, full efficacy and potency 10 times higher than the triazolyl benzotriazole derivative bearing an H atom at position 5. The activity of the latter compound is significantly reduced by high levels of KCl (60 mM), suggesting a possible pharmacodynamic profile similar to that operated by drugs known as activators of potassium channels [105]. Finally, another series of 5-substituted-1-(2-hydroxybenzoyl)-benzotriazoles, wherein the triazole group is replaced by a hydroxybenzoyl group, (Fig. 15, series III); thereby, thanks to the bridge determined by the carbonyl function, the new molecules were ensured by a greater degree of freedom and therefore flexibility between the two main parts. Biological analysis shows that all these derivatives show high vasorelaxing potency and efficacy. However, once again, the more potent derivative in the context of this set appears to be the one having, at 5-position of the benzotriazole moiety, the methyl group; this compound also has cardioprotective properties. In contrast, both the introduction of the 2-hydroxyphenyl group in position 1 of the benzotriazole ring and the replacement of the carbonyl bridge (an electron withdrawing group) with a methylene bridge causes a considerable decrease of the biological activity [106].

BENZOTRIAZOLE AS TUBULIN POLYMERIZATION INHIBITOR

Cancer is actually the second leading cause of death worldwide after cardiovascular diseases, accounting for about 8 million deaths (around 13% of all deaths) [107, 108]. The origin of a tumor resides in a somatic cell that has undergone a series of genetic modifications, thus ceasing to respond to normal regulatory mechanisms that operate in a healthy organism. This cell then proliferates to form a clone of neoplastic cells, characterized by lack of control proliferation. At the same time the cell also lose control of cellular replicative potential, and becomes immortal. Low is also the mechanism for density-dependent inhibition, which, in normal conditions, allows the cells to multiply up to a defined cell density, at which they become quiescent. The tumors can be distinguished in benign and malignant, depending on the biological and morphological characteristics that determine a greater or lesser aggressiveness. The most common cancers are lung cancer (1.4 million deaths), stomach cancer (740.000 deaths), liver cancer (700.000 deaths), colorectal cancer (610.000 deaths) and breast cancer (460.000 deaths) [109].

The therapeutic approach for the treatment of cancer diseases may be different, and includes:

surgical treatment, radiation therapy, immunotherapy, chemotherapy. Polychemotherapy therefore provides the use of virtually toxic drugs, which operates selectively in respect of cancerous cells, thus saving the healthy cells of the host [110]. Unfortunately, so far this selectivity is practically unattainable due to the difficulty in discriminating between tumor and healthy cells, these being very similar to each other. All this translates into an exposure to the toxic effects on the part of all healthy somatic cells, especially those in more rapid replication, such as those of the hair follicles, intestinal epithelium and bone marrow [111].

For this reason, the research for new molecules able to present a selective toxicity against specific types of cancer is still present. Nitrogen heterocycles, in particular, attracted the attention of researchers as possible isosteres of structural components of natural nucleotides [112-114], and also benzotriazole has been reported as possible antiproliferative agent [85, 115, 116].

This principle of isosterism was followed by Zhan and Lou [117] in the preparation of new nucleoside analogues with antitumor activity. Previously it had been shown that the carbocyclic nucleosides (obtained by replacing oxygen with a sugar-CH_2 group) are more stable to the action of nucleoside phosphorylase and are also equipped with anti-tumor activity [118-120]. On this basis, Zhan and Lou decided to further modify the structure of the nucleoside analogues, replacing the heterocyclic base with various polinitrogen heterocycles. The different analogues of D-pinitol were tested on tumor cell lines derived from human lung and bladder, but only derivatives with substitution of the triazole and benzotriazole were active, albeit at micromolar level, as shown in Fig. 16.

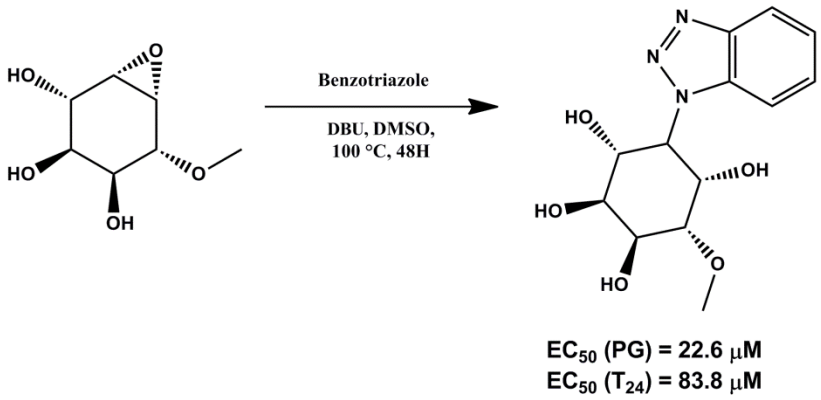


Fig.16. 3′-O-Methyl-5′-deoxy-5′-(1,2,4-triazole-1-yl)-D-chiro-inositol.

On the same principle worked Al-Soud's research group, combining the potential reported for several alkylated benzotriazoles with that of 1,2,4 triazole nuclei (Fig. 17). Starting from 1 and 2 (cyanomethyl)benzotriazole were prepared azepinic compounds, which, as result from biological evaluation, were found to be extremely promising. From the analyzes performed on several human tumor cell lines, the compound 2-((1*H*-benzo [*d*] [1,2,3] triazol-1-yl) methyl) -6,7,8,9-tetrahydro-

5*H*-[1,2,4] triazolo [1,5-*a*] azepine stands out for its micromolar activity against tumor cells derived from leukemic (CCRF-CEM and RPMI-8226, GI_{50} = 0.07 mM), ovarian (OVCAR-3, GI_{50} = 0.02 mM) and renal (CARKI-1, GI_{50} = 0.06 mM), although not showing selective in its action. In contrast, the isomer of substitution at N-2 demonstrated no activity [121].

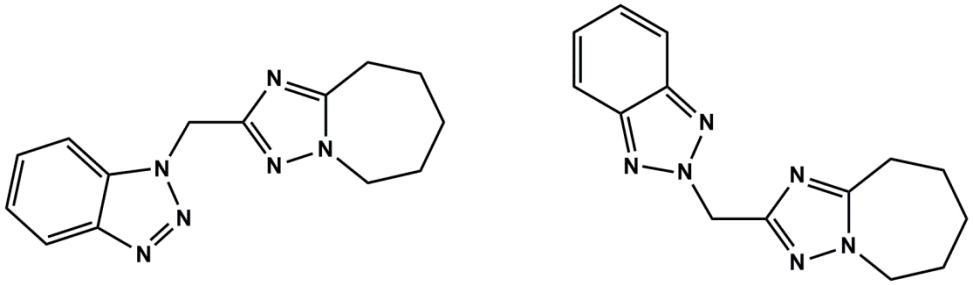


Fig. 17. Benzotriazol-1(2)-yl)-tetrahydro-triazoloazepine.

The interest generated by the indoloquinolinic alkaloids, i.e. Cryptosanguinolentine [122, 123], has instead pushed the group coordinated by Beauchard to design and prepare a series of derivatives in which the thiazole ring system is fused in polycyclic systems. They prepared a series of new thiazole-benzotriazoles as synthesis intermediates, all of which are evaluated as potential anticancer agents [124]. Starting from 2-cyano thiazole-benzotriazoles [24], for substitution on the nitrogen *N*-1 and *N*-3 with quinoline and pyridine nuclei, were prepared compounds whose reactivity of the cyano group in the 2-position of the benzothiazole has been exploited in order to prepare imidazoline derivative, obtained by reaction with ethylenediamine, as shown in Fig. 18.

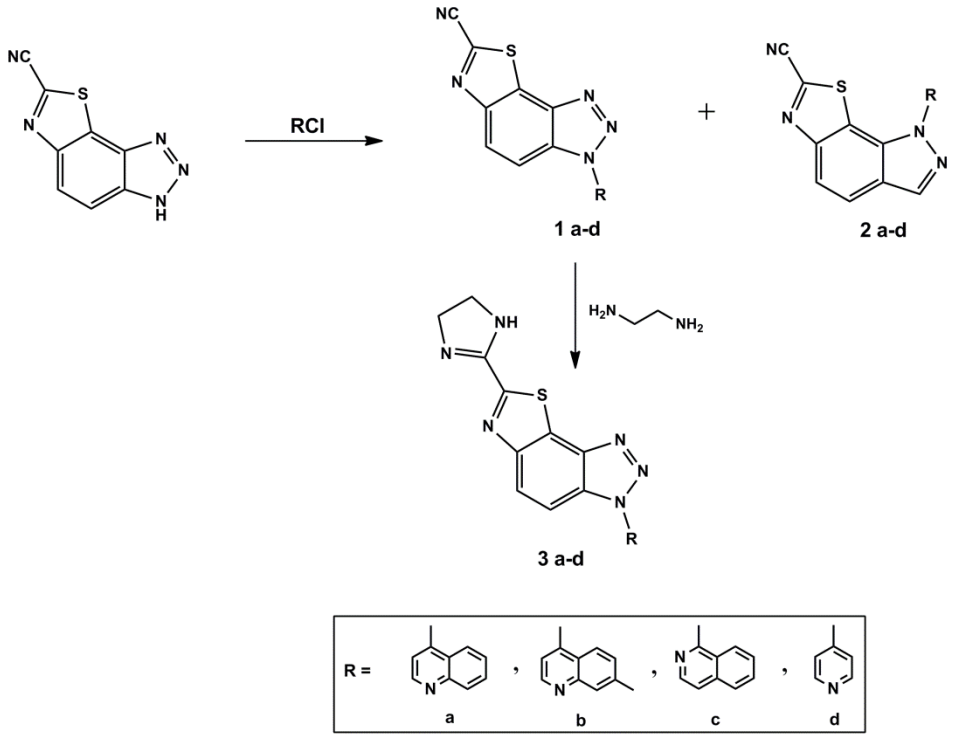


Fig. 18. Cyano thiazole-benzotriazoles derivatives.

All compounds of substitution at *N*-1, tested on two cellular lines derived from breast cancer, were found to be from moderately to very active (% inhibition between 32.4 ± 1.0 for **1a** derivative, and 97.4 ± 1.0 for **2b** on MDA-MB- 231, the most resistant cell line) with the exception of compounds **1c** and **3c**, inactive on both lines. Generally the introduction of an imidazolinic side chain determines an increase of the biological activity, except for the compound **3d**.

Finally, the group of Wan and co-workers has long been devoted to the evaluation of the benzotriazole derivatives antiproliferative activity [125-127]. 3-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)-1-(4-methoxyphenyl)-1-oxopropan-2-benzoate (BmOB) emerged for its antiproliferative effect on cell lines of different tumor type. This molecule was therefore used in order to define a possible mechanism of action. The analyzes carried out on BEL-7402 hepatocellular carcinoma cells, the most susceptible to BmOB action (IC_{50} = 0.082 ± 0.008 mM), show that this molecule can lead to cell death through induction of a collapse of mitochondrial membrane potential, determining both the production of reactive oxygen species and DNA fragmentation [128].

In later studies, exploiting the concept of bioisosterism, on known 3-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)-2-bromo-1-phenylpropan-1-onic intermediates [129, 130], were introduced similar and bulky side chains by reaction with nicotinic or isonicotinic acid. The 3-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)-1-(4-chlorophenyl)-1-oxopropan-2-nicotinate (A) and 3-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)-1-oxo-1-p-tolilpropan-2-nicotinate (B), shown in Fig. 19, demonstrate to possess potent propagation inhibition activity in liver and galactophore cancer cells [131].

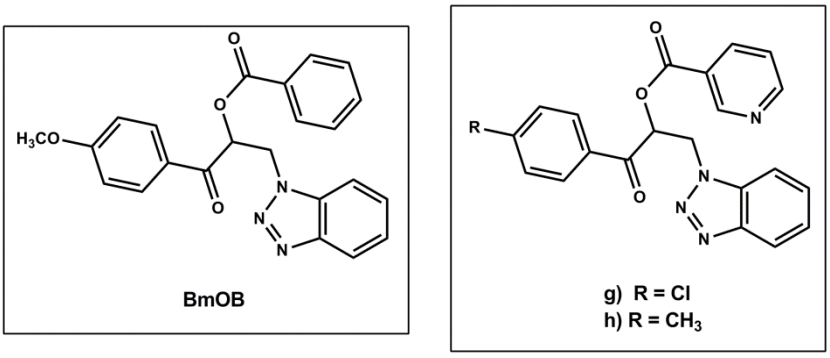


Fig. 19. Chemical structure of BmOB and nicotinic derivatives **g** and **h**.

Finally, as part of a wide study focused on benzotriazole derivatives that could act against *M. tb*., Carta and coworkers discovered that compounds belonging to the class of 3-Aryl-2-[1*H*-benzotriazol-1-yl]acrylonitriles were able to inhibit cellular proliferation in a series of liquid and solid human tumors [10, 83, 84].

Principally, researchers identified (*E*)-2-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)-3-(4-methoxyphenyl) acrylonitrile as lead compound, shown in Fig. 20, found to be 5 to 100 times more potent than 6-mercaptopurine and comparable to etoposide on all cell lines [85, 115]. Similar derivatives

possessing in the benzotriazole positions 5 and 6 electron-donor substituents, such as methyl groups, results to be absolutely devoid of efficacy on the same tumor lines.

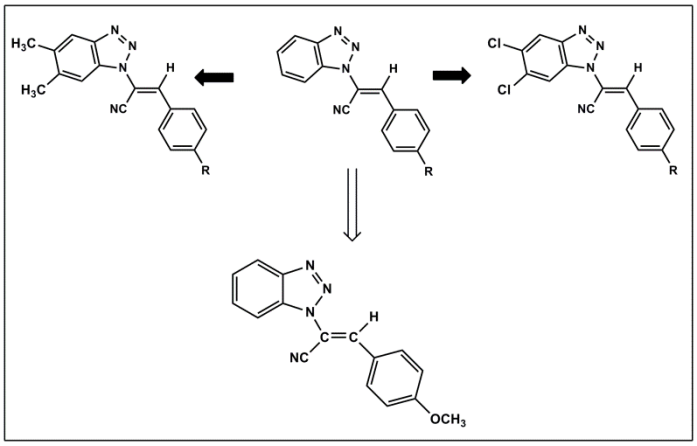


Fig. 20. Benzotriazol acrylonitriles: structure and lead compound of the series, (*E*)-2-(1*H*-benzo [*d*][1,2,3]triazol-1-yl)-3-(4-methoxyphenyl)acrylonitrile.

Therefore, in 2011, the group completed the SAR studies, designing and synthesizing molecules possessing at position 5 and 6 electron-withdrawing group, such as chlorine. However, the derivatives (*E*)-2-(5,6-dichloro-1*H*-benzo[*d*][1,2,3]triazol-1-yl)-3-(4-R-phenyl)acrylonitriles [115] resulted almost devoid of activity, demonstrating that the introduction of a chlorine atom in the benzotriazole moiety determines a considerable decrease of the antiproliferative activity for this class of derivatives. The cell cycle analysis also revealed that this series of compounds act at the G2/M phase of the cell cycle and this result can be explained by a possible interaction of the derivative with tubulin [132, 133].

This result was further confirmed by [^{3}H]Colchicine competition-binding scintillation proximity assay (SPA) [134], in which one of the derivatives of benzotriazole displaces strongly colchicine radio-labeled from its binding site on tubulin. It is also reported an extended molecular modeling study carried out using a model of tubulin obtained by homology from Bos taurus β-tubulin, which shows a 98% sequence homology with the human one, Fig. 21. This model has allowed to thoroughly study the binding site and the molecular interactions that exist between the *E*-3-aryl-2-(1*H*-benzotriazol-1-yl)acrylonitriles and the amino acidic residues of the binding pocket.

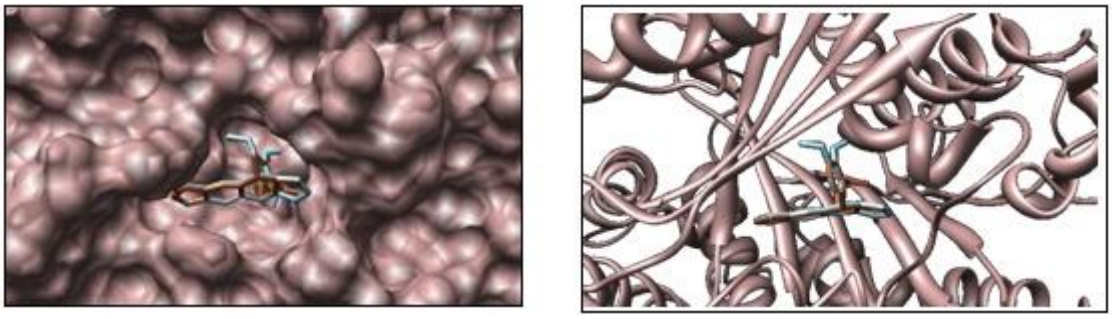


Fig. 21. Validation of the three-dimensional model. Comparison of co-crystallized podophyllotoxin

(brown) and subjected to docking (blue) in the allosteric binding site on human β-tubulin.

From this type of molecular analysis, and as shown in Fig. 22, it emerge that the benzotriazole ring interacts with tubulin mainly through Van der Waals interactions and that the compounds act inserting the triazolic moiety in the colchicine binding site. Consequently these compounds inhibit tubulin polymerization acting as microtubules destabilizing agents.

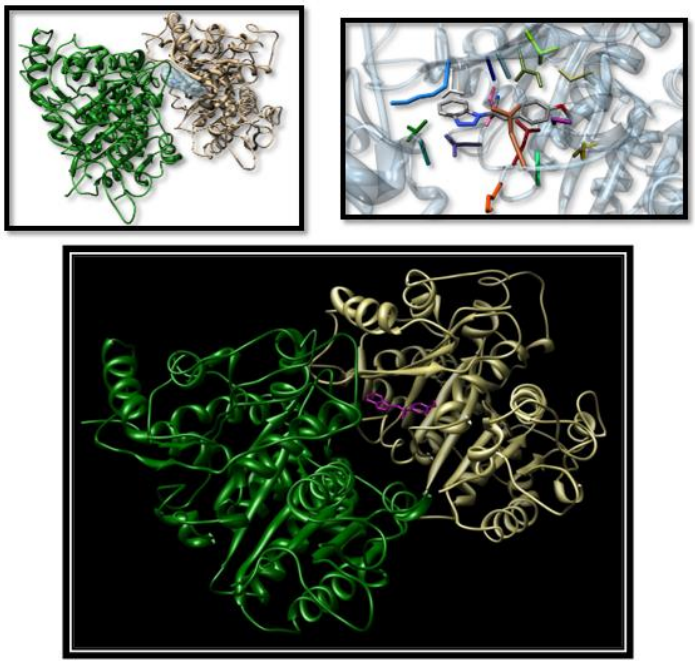


Fig. 22. Interaction of the derivative (*E*)-2-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)-3-(4-methoxyphenyl)acrylonitrile in the binding site of tubulin.

BENZOTRIAZOLE AS PROTEIN KINASE CK2 INHIBITOR

The Casein kinase 2 (CK2) is a pleiotropic protein highly conserved, a serine/threonine and tyrosine kinase constitutively active [135] involved in numerous metabolic processes. It is believed that the CK2 is involved in cell growth, both in healthy and cancerous cells [136], in the synthesis of tRNA and mRNA [137], also in apoptosis and in cell transformation. The CK2 has been described for the first time by Burnett and Kennedy [138] and different isoforms were identified [139, 140]. However, the CK2 conventionally exists either in form of tetrameric complexes, composed of two catalytic subunits (α and α', or α'') [141-143] and two regulatory subunits (β and β'), or as free subunits [144, 145]. There are also indications that tetrameric CK2 can be assembled into more complex structures. There is increasing evidence to suggest that different molecular forms of CK2 may have different cellular functions. For instance, some substrates of CK2 can only be phosphorylated by tetrameric CK2, while others are phosphorylated exclusively by the free catalytic subunits [146]. Also, have been identified proteins able to interact with CK2, and some of these

discriminate between tetrameric and CK2 as individual subunits [147].

Scientific literature demonstrate that the CK2 has antiapoptotic properties, being able to interfere with important component of cell survival pathways, especially by determining down-regulation of pro-apoptotic proteins such as caspases [148]. More papers shown that the evolution of neoplastic disease and the onset of cancer is directly proportional to the CK2 activity [149]. Furthermore, it was demonstrated that the CK2 shows oncogenic activity in transgenic mice and is frequently over-expressed in tumor and leukemia cells [150-152]. Finally, the high constitutive activity of CK2 is suspected to be behind its pathogenicity potential, since it is exploited by several viruses to exert the phosphorylation of proteins essential to their life cycle [153]. Furthermore, the catalytic subunit α cooperates with other protooncogenes to promote cell transformation in different experimental models [150]. This makes CK2 an attractive target in the search for new antineoplastic and antiviral agents.

In recent years, potent and selective CK2 inhibitors have been developed; among these are the 4,5,6,7-tetrabromobenzimidazole (TBBi) and 4,5,6,7-tetrabromobenzotriazole (TBBt), shown in Fig. 23.

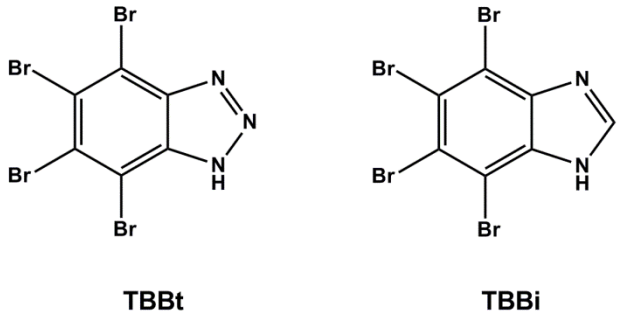


Fig. 23. 4,5,6,7-tetrabromobenzimidazole (TBBi) and 4,5,6,7-tetrabromobenzotriazole (TBBt) chemical structure.

Sarno and co-workers showed that TBBt is the most selective and specific inhibitor of CK2 among those so far analyzed. Among the 33 protein kinases tested by this research group, only three are inhibited by TBBt and their respective IC_{50} values appear to be about two orders of magnitude higher than the value calculated using the CK2. The three kinases inhibited by TBBt are CDK2 and GSK3L, belonging to the same protein kinase subfamily (named CMGC group), and PHK , belonging to the CaMK group. Moreover, it was shown that, within the same CK family, TBBt turns out to be more selective for CK2 (Ki = 0.4 μM), compared with CK1 (Ki = 47 μM) [154]. A third class of CK proteins, localized to the Golgi apparatus (G-CK), were instead totally refractory to TBBt [155]. All this suggests that TBBt possess a particular selectivity only towards CK2 as inhibitor agent. This property, combined with its ability to penetrate inside the cell and lack of

evident short term cytotoxicity, makes the TBBt a promising leads to design new compounds having a high therapeutic potential .

Zien research group compared the ability of action of TBBt and TBBi against CK2 purified holoenzymes, showing that the latter is not only far more effective in discriminating between different forms of CK2 present in yeast [156, 157], but is also dramatically more effective at inducing apoptosis and, to a lesser extent, necrosis, in transformed human cells [158]. Moreover, the solution of the crystal structure of the complex between CK2 and 4,5,6,7-tetrabromo-1*H*-benzimidazole [159] has allowed a comparison with the structures of the complexes with 4,5,6,7-tetrabromo-1*H*-benzotriazole [160] leading to the conclusion that despite their structural similarities they binds the biological target in a quite different manner.

The history of TBBt born in 1950, when it was demonstrate that the 1-α-ribofuranosido-5,6-dimethylbenzimidazole (Fig. 24**)** represents a key constituent for vitamin B_{12} [161, 162]. This has subsequently stimulated the synthesis of a variety of structural analogues to evaluate their biological activity, particularly on cell proliferation. It was then discovered that the 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB) is able to inhibit the mRNA synthesis in eukaryotic cells, in *vivo* and *in vitro* [163]. Additionally 5,6-dibromo-1-β-D-ribofuranosylbenzimidazole is a more potent inhibitor of *in vivo* and *in vitro* transcription, inhibiting at the same time purified CK2 activity at 6-10 times lower concentrations than DRB [164].

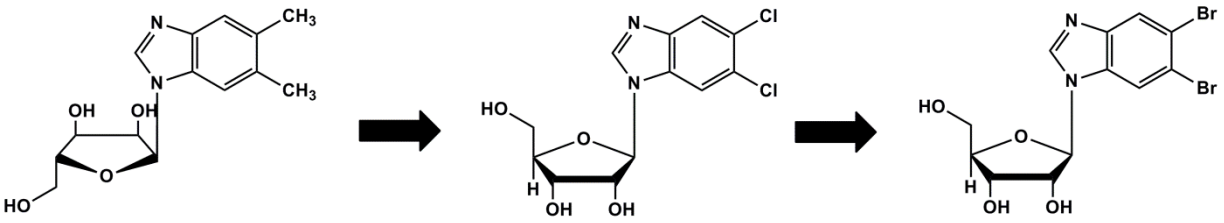


Fig. 24. 1-α-ribofuranosido-5,6-dimethylbenzimidazole derivatives.

All these works stimulated the study of other halogenated benzimidazole nucleoside analogues [146, 165] and led eventually to the synthesis of TBBt [166]. The specificity of inhibition of TBBt was then exploited extensively to understand the functionality of the CK2. The research group of Battistutta, for instance, studied the interactions between the TBBt and *Zea mays* protein kinase CK2α catalytic subunit. Particularly, the enzyme-inhibitor complex crystal structure has been delineated, highlighting that the specificity and selectivity of TBBt for CK2 appears to be mainly dictated by the reduced size of the active site, a small hydrophobic pocket adjacent to the binding site of ATP/GTP, in which TBBt fit perfectly, as depicted in Fig. 25.

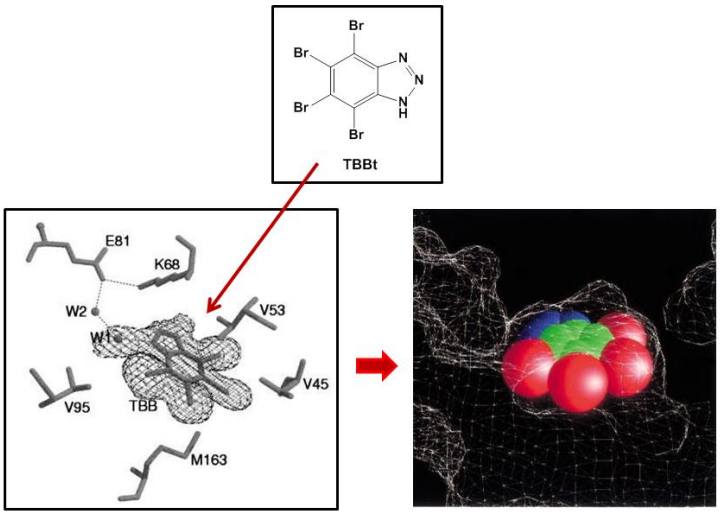


Fig. 25. TBBt inside CK2α hydrophobic binding pocket. From: *Protein Sci*. 2001 November; 10(11): 2200–2206. © Copyright 2001 The Protein Society.

The main interactions between TBBt and CK2 are hydrophobic in nature and involve the four bromine atoms, whose size makes it the perfect insertion into the cavity and at the same time hinder the release of the inhibitor, once it has been trapped inside the pocket [160].

The inhibitory power decreases if the four bromine atoms of TBBt are replaced with smaller atoms, for instance with chlorine atoms, as previously observed by the Szyszka's research group [166]. It therefore seems that the TBBt lose its inhibitory potency against the majority of protein kinases, simply because the hydrophobic binding site in most other protein kinases is generally too large for making stable interactions with this inhibitor.

The relevance of the individual bromine atoms of 4,5,6,7-tetrabromobenzotriazole (TBBt) was then investigated by the group of Wasik, which synthesized all the possible isomers of analogs TBBt, mono-, four di-, and two tri-bromobenzotriazoles, evaluating the physico-chemical properties in an aqueous medium and confirming that hydrophobic and electrostatic interactions are predominant in halogenated benzotriazoles, determining a selective activity inhibition on protein kinase CK2α [167].

In order to increase the TBBt and TBBi CK2 inhibitory activity, the research groups of Bretner and Najda-Bernatowicz designed new tetrabromo derivatives in order to evaluate the effect of alkyl substituents with different hydrophobic, steric and electrophilic features attached to TBBt and TBBi on their inhibitory potency. Their research program is based on the evidence that water molecules, necessary for enzymatic activity, are coordinated at the binding site on CK2 [159], molecules that could be displaced by similar substituents. New N-hydroxyalkyl derivatives were then tested on human CK2 holoenzyme ($\alpha_2\beta_2$), demonstrating that the pharmacological activity depends on the length of the alkyl chain. Some of the N-hydroxyalkyl derivatives showed IC_{50} values similar to that

of the parent compounds, and best results were ensures by the propan-1-olic chain, with IC_{50} values of 0.48 μM [168]. In 2009, the group investigated the effects of bulkier alkyl chains, with both hydrophobic and polar properties. Once again, the 3-(4,5,6,7-tetrabromo-1*H*-benzimidazol-1-yl)propan-1-ol (**I**) appears to be the best in the series, with small differences from the N2-substituted derivative (**II**) (Fig. 26). On the contrary, derivatives with shorter or longer chains see a drastic decrease in their activity. Additionally they demonstrated that the four bromine atoms in the benzene ring seem to be an essential requirement for biological activity. Indeed, the substitution of bromine with chlorine or CH_3 groups leads to significantly lower inhibitory effect versus different forms of CK2, human and not. The relevance of the triazole system is finally put in evidence with its replacement with a phthalimide moiety, present in many biologically active compounds [169, 170], or phthalazine system. The derivatives thus obtained results in fact powerless [171].

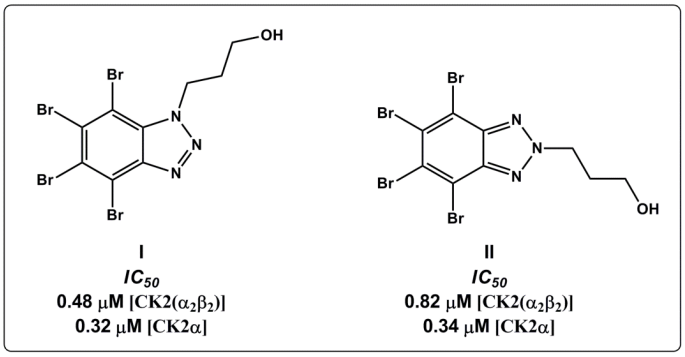


Fig. 26. TBBt N-hydroxyalkyl derivatives.

Other research groups continued to design new TBBt derivatives in consideration of the fact that the hypothesis concerning the small size of the TBBt binding site on CK2 has faded over the years [172], especially in the light of the biological successes reported by dimensionally bigger molecules [173, 174].

Were then conducted new researches introducing on the triazolic moiety even bulkier side chains. Was also evaluated a modification of the tetrabrominate benzene ring, replacing the two bromine atoms in 4 and 5 with aryl and alkyl substituents. Unfortunately it was not found any improvement in activity compared to TBBt inhibitory potency (IC_{50} = 0.46 mM), except for 5,6,7-tribromo-4-methyl-1*H*-benzotriazole (IC_{50} = 0.51 μM) and 5,6,7-tribromo-4-ethyl-1*H*-benzotriazole (IC_{50} = 0.16 μM), as shown in Fig. 27 [175].

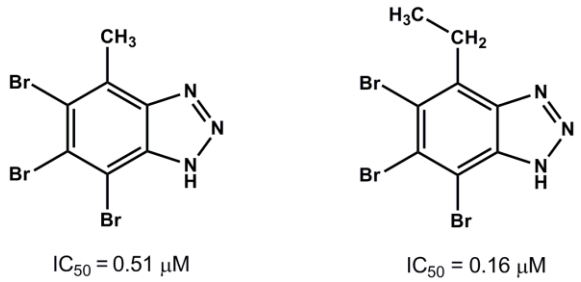


Fig. 27. 5,6,7-tribromo- 4-methyl-1*H*-benzotriazole and 5,6,7-tribromo-4-ethyl-1*H*-benzotriazole chemical structures.

The Swider group instead synthesized new benzotriazole derivatives, designed using a receptor-based design approach to interact simultaneously with the Mg^{2+}-chelating residues and the protein-substrate binding residues [176]. From the synthetic point of view, these compounds were obtained by Azide-alkyne Huisgen cycloaddition on tetrabromobenzotriazole derivatives. The result is a combination of the tetra-halogenated moiety, which nicely occupies the ATP-binding, with side chains able to bind simultaneously both the enzymatic active site and the basic residues that participate in protein substrate binding, like similar bisubstrate inhibitors of other kinases [177]. In this paper were reported the preliminary and non-optimal results for the most active compound, 4-(4-(2-(perbromo-2*H*-benzo[*d*][1,2,3]triazol-2-yl)ethyl)-1*H*-1,2,3-triazol-1-yl)butan-1-amine (IC_{50} μM 6.33 ± 0.23), which synthesis is reported in Fig. 28.

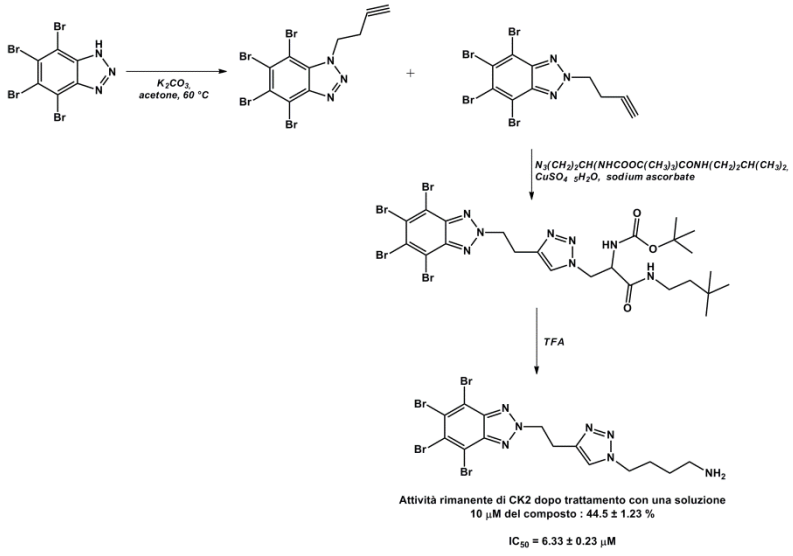


Fig. 28. 4-(4-(2-(perbromo-2*H*-benzo[*d*][1,2,3]triazol-2-yl)ethyl)-1*H*-1,2,3-triazol-1-yl)butan-1-amine synthesis.

Finally, it is important to report the new synthetic approach of Wawro and co-workers, which is based on two considerations: 1) none of the previously synthesized halogenated derivatives of TBBt had a *chiral* center, and 2) the presence of a stereocenter close to a polar group, i.e. an OH, could lead to a different inhibitory activity in the two enantiomers. Based on these considerations and

taking as reference the hydroxyalkyl derivatives synthesized by Nadja-Bernatowicz [171] and Makowska [175], the group has developed a new synthetic approach to isolate enantiopure hydroxyalkyl derivatives of 4,5,6,7-tetrabromo-1*H*-benzotriazole, obtaining optically active compounds endowed with enantioselectivity factor (*E*) >200 [178]. These molecules, potential CK2 inhibitors, might be used to synthesize new, optically pure TBBt derivatives.

BENZOTRIAZOLE IN COORDINATION COMPOUNDS

As above, the benzotriazole coordination complexes showed antiproliferative activity [69]. A study on *N*-(4,5-diidroimidazol-2-yl)azoles derivatives, involved in coordination complexes with transition metals, has highlighted their potential anticancer properties [179]. On this basis, the group of Saczewski has reported the synthesis and biological evaluation of some chelating bidentate benzotriazole Copper(II) Complexes [180]. Such complexes have been designed with the idea to create compounds endowed with potential copper-zinc-superoxide dismutase (Cu, Zn-SOD) mimicking activity. This activity in tumor cells, according to the theory of Oberley and Buettner, is less than that found in normal cells [181]. Were therefore analyzed the [2-(4,5-dihydro-1*H*-imidazol-2-yl)-1*H*-benzotriazole]-dichlorocopper(II) and [1-(4,5-dihydro-1*H*-imidazol-2-yl)-2*H*-benzotriazole]-dichlorocopper(II) complexes results possess the lower and the higher SOD activity respectively. Since these compounds differ only in the substitution position to the benzotriazole ring, it follows that the biological activity is exquisitely sensitive to the structure of the coordinating ligand (Fig. 29). The derivative of substitution in 2 showed a potent *in vitro* SOD activity, with 0.06 μM IC_{50} value, comparable with that reported in literature for other agents mimicking SOD. Evaluating the *in vitro* cytotoxicity on seven tumor lines, once again the derivative of substitution in 2 turns out to be the best one, recording an IC_{50} values between 13 and 28 μM.

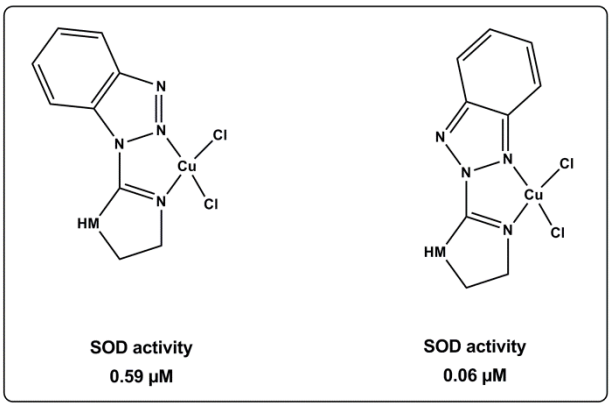


Fig. 29. Chelating bidentate benzotriazole Copper (II) complexes.

BENZOTRIAZOLE AS HISTONE DEACETYLASE INHIBITOR

The histone deacetylase (HDAC) catalyzes the deacetylation of lysine residues, especially at the

histones H3 and H4 level [182]. This chemical modification is a key step in the regulation of genes expression that influence cell differentiation and proliferation processes [183]. An abnormal functioning of the HDACs has been linked to the carcinogenesis process [184]. It was found that many compounds able to inhibit HDACs can exert an antitumor effect *in vivo* [185].

There are eighteen humans HDAC and these enzymes are divided into four classes, according to their homologies to yeast's HDAC, to the subcellular localization and to the enzymatic activity. Class I includes HDAC1, 2, 3 and 8; class II is composed of six members, HDAC4, 7, 9 and 10; class III, also known as sirtuines, includes SIRT1-7, which are NAD(+)-dependent enzymes; class IV, which includes HDAC11, has the properties of both HDACs class I and class II [186]. HDAC inhibitors can be classified into five groups, according to their chemical structure: 1) hydroxamic acids, 2) cyclic tetrapeptides (apicidina); 3) short chain carboxylic acids, such as valproic acid; 4) benzamides 5) ketoacids [187].

However, in a recent paper by Fu and co-workers, even benzotriazole derivatives have been recognized as antiproliferative agents that exert their biological action through inhibition of histone deacetylase. In particular, the group synthesized various benzotriazole compounds bearing substituted benzoic acids, whose antitumor activity was evaluated on three different human tumor cell lines. All compounds showed antiproliferative activity and IC_{50} values between 1.2 and 666 nM. Among these compounds 1*H*-Benzo[*d*][1,2,3]triazol-1-yl 3,4,5-trimethoxybenzoate stands out with an IC_{50} value of 1.2-2.4 nM, very close to that of positive control, doxorubicin. The SAR analysis emphasized the relevance of the OCH_3 group for the antiproliferative activity: in fact derivatives bearing such substituents at benzoic ring position 3, 4 and 5 are the most powerful, while methoxyl substitution of benzotriazole group results in a drastic reduction of antiproliferative activity [188]. The HDAC inhibitory activity was determined using HeLa nuclear extract as the enzyme source, taking trichostatin (TSA) and suberoylanilide hydroxamic acid (HSA) as reference drugs. From the obtained results it is clear that the more potent derivative turns out to be once again trimethoxy derivative, in agreement with the result obtained for the antiproliferative activity. From this we can deduce that this activity is related to the ability of the compound to inhibit HDAC. This derivative was finally subjected to studies of molecular docking to simulate its interaction with the HDAC and to studies on the binding model based on the crystal structure of HDLP extracted from the HDLP/TSA complex using the AUTODOCK 4.0 software [189]. From these researches it appears that there are some kinds of hydrophobic interactions of the benzotriazole and phenyl moieties with Phe141, Tyr196, Leu265, Lys267, Tyr297 of the enzyme (Fig. 30).

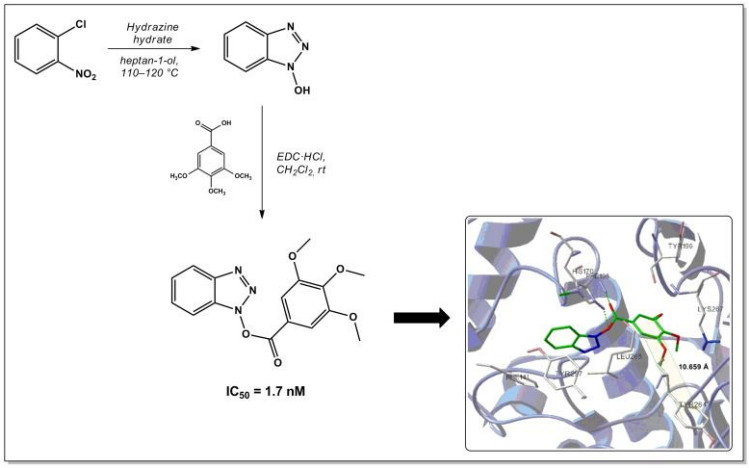


Fig. 30. 1*H*-benzo[*d*][1,2,3]triazol-1-yl 3,4,5-trimethoxybenzoate synthesis and binding mode.

BENZOTRIAZOLE ALKANOIC ACIDS DERIVATIVES AS PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPARS) AGONIST.

The peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of intracellular receptors regulate many biological processes, including energy metabolism (glucose and lipid metabolism), cell proliferation, skin development and inflammatory process [190, 191]. The PPARγ is a member of the second class of the steroid nuclear receptors superfamily and regulate gene transcription through the formation of functional heterodimers with retinoid X receptor *(*RXR*)* for 9-cis-*r*etinoic acid. The PPAR/RXR complex modulates the expression of target genes by recognizing specific responsive sequences consist of a direct repeat (DR) of a hexanucleotide sequence [5'-TGA(A/C/T)CT] separated by a single nucleotide (DR1) [192].

The PPAR subfamily includes three isoforms: α, β/δ, and γ. Encoded by different genes, only for the PPARα and PPARγ specific ligands have been identified consisting of saturated and polyunsaturated long-chain fatty acids, eicosanoids, and h*ypolipidemic* agents. In particular, among PPARα agonists we can remember the fenofibrates, gemfibrozil, bezafibrate and clofibrate, all developed as drugs for the treatment of dyslipidemia, whereas pioglitazone and rosiglitazone are agonists of PPARγ and have been developed as drugs for the treatment of *type 2 diabetes mellitus [193]*. However, neither fibrates nor glitazones are able to simultaneously lowering triglycerides and glucose blood levels; therefore PPAR agonists research is thus ever timely, useful for the treatment of dyslipidemic patients suffering from type 2 diabetes [194, 195].

According to studies conducted by the Sparatore research group, many PPAR agonists possess three key regions: a) an acid head (a thiazolidinedione ring or an alkanoyl residue); b) a linker portion (a benzene ring bearing variously functionalized chain in the para position to the head); c) an hydrophobic tail (formed by a mono- or polycyclic aromatic or heteroaromatic moiety).

Thus, to develop new PPAR α, β/δ , and γ activators, Sparatore *et al.* decided to stiffen the last two parts, suppressing the flexible chain linker between the aromatic rings. Following this way a series of [4-(2*H*-1,2,3-benzotriazol-2-yl)phenoxy]alkanoic acids was synthesized [196]. The 4-(2*H*-1,2,3-benzotriazol-2-yl)phenoxy moiety, which characterizes these compounds, is also present in the 5-chloro-2-(4-[3-(dimethylamino)propoxy]phenyl)-2*H*-1,2,3-benzotriazole, molecule previously recognized able to reduce significantly the level of cholesterol in hypercholesterolemic mice, measurement comparable to that of bezafibrate [197]. Undergoing biological tests, the study shows that the compounds 3-(4-(2*H*-benzo[*d*][1,2,3]triazol-2-yl)phenoxy)-2,2-dimethylpropanoic acid and 3-(4-(2*H*-benzo[*d*][1,2,3]triazol-2-yl)-2,6-dimethylphenoxy)-2,2-dimethylpropanoic acid, dual activators of PPAR α/δ and PPAR α/γ, respectively, possess an effectiveness equal to that of the reference compound, the Wy-14643 [198], but differ strongly in potency, presenting respectively EC_{50} of 10.5 μM and 96 nM ($EC_{50\ \text{Wy-14643}}$ = 9.3 μM) (Fig. 31).

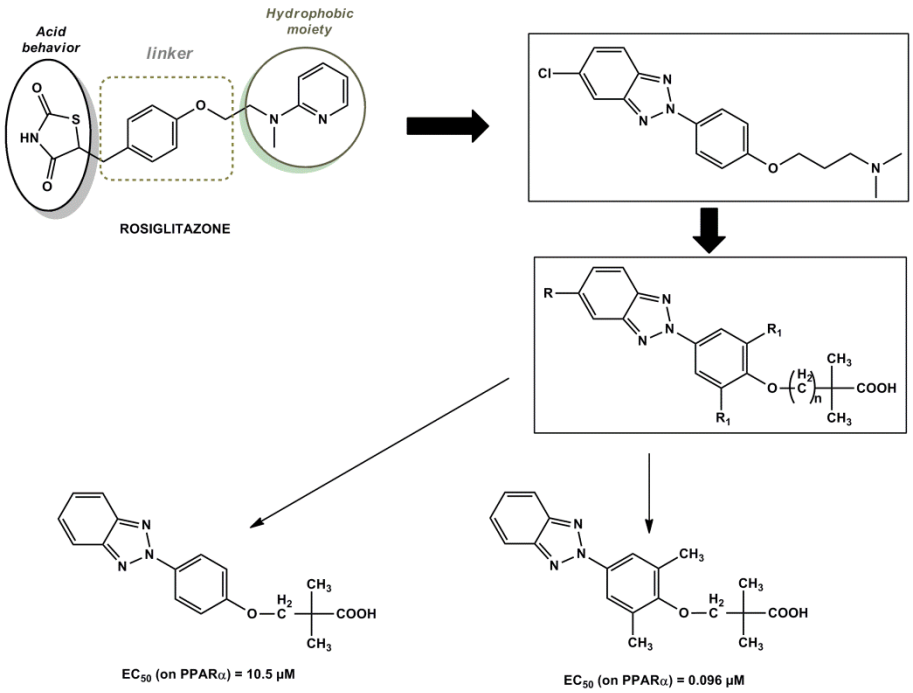


Fig. 31. Benzotriazol-2-yl-phenoxy-2,2-dimethylpropanoic acid derivatives.

Interestingly, the 5-chloro-2-(4-[3-(dimethylamino)propoxy]phenyl)-2*H*-1,2,3-benzotriazole is found to be inactive on all three receptors at concentration up to 100 μM, demonstrating that probably the lipid-lowering effect of these molecule is due to a different mechanism of action from the activation of PPAR.

In summary, the obtained results show that the 4-(2*H*-1,2,3-benzotriazol-2-yl)phenoxy moiety that characterizes these compounds represents a new scaffold for the synthesis of potential PPAR agonists for the treatment of dyslipidemia associated with type 2 diabetes (NIDDM) or particular kinds of dyslipidemia not associated with diabetes.

BENZOTRIAZOLE AS LIGAND FOR SEROTONIN AND DOPAMINE RECEPTORS

Is well known that the neurotransmitter 5-hydroxytryptamine (5-HT), more simply called serotonin, is implicated in numerous physiological functions, including control of appetite, regulating mood, voluntary movement , sleep body temperature, attention, memory and learning [199-201]. Alteration in the serotonergic system may then determine psychiatric disorders such as anxiety , depression and obsessive-compulsive disorder (OCD) [202]. For this reason, dopamine and serotonin agonists and antagonists have been used in recent years for the treatment of schizophrenia and Parkinson's diseases [203-205].

The Caliendo's research group reported a series of 1/2-[3-(4-(x)-1-piperazinyl)alkyl]-benzotriazoles derivatives, designed as structural analogues of trazodone, known psychoactive drug belonging to the piperazine and triazolopiridine class. The synthesized derivatives, as shown in Fig. 32, contain three major structures:

1) a N-4 substituted piperazine ring;

2) a cyclic system containing a substituted benzothiazole nucleus;

3) an alkyl or alkyloxy linker which acts as a *trait d'union* between the piperazinic and benzotriazole moiety.

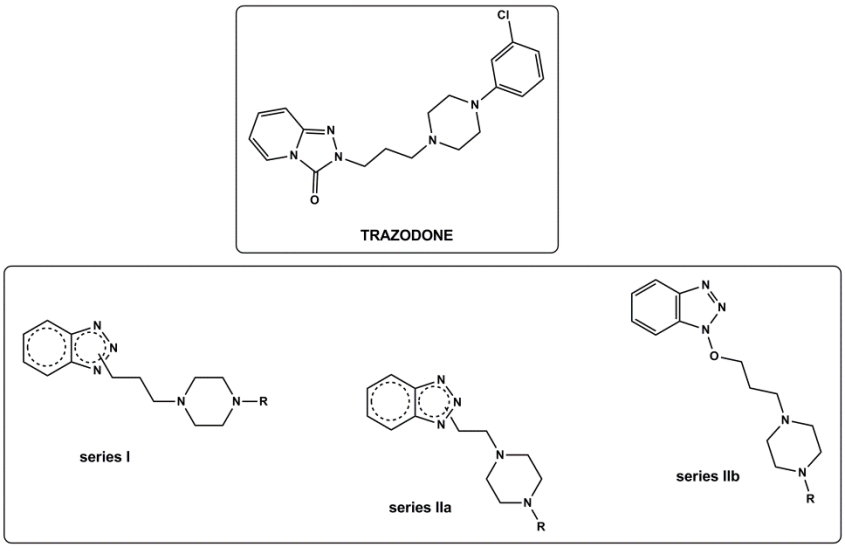


Fig. 32. 1/2-[3-(4-(x)-1-piperazinyl)alkyl]-benzotriazoles derivatives.

For these derivatives were evaluated *in vitro* the anti-serotonin, anti-adrenergic and anti-histaminergic effect, but also *in vivo* the analgesic activity [206, 207]. Concerning the anti-serotonin activity, the results obtained show that the most powerful derivatives belong to the 1-(2-(4-(R)-1-piperazinyl]-ethyl- benzotriazoles series, where R = Ph, 2-Cl-Ph, 3-Cl-Ph, 4-Cl-Ph, CH_2-Ph, Me, CH_2-CH_2-OH. Derivatives bearing alkyl substituents on the 4-piperazine nitrogen showed no or

extremely low activity. Moreover, the introduction of a chlorine atom at the ortho and meta positions of the phenyl ring determines an increase of activity, while at the para position determine a loss of it by 10 to 100 times. Still, the introduction of a methylene or ethylene bridge between the aromatic ring and the piperazine nucleus leads to a decrease in activity due to a steric and electronic effect, rather than to a hydrophobic effect.

With regard to the anti-adrenergic activity, has been evaluated the *in vivo* ability to block the noradrenaline-induced contraction of rat vas deferens. The most active compounds were derivatives 1- and 2-[3-[4-(x)-1-piperazinyl]-propyl]-benzotriazoles (x = Ph, 4-Cl-Ph).

The anti-histamine effect was instead determined for compounds presenting simultaneously at the side chain an oxypropylene bridges and an aromatic moiety. The substitution of the aryl or benzyl group with a methyl or a β-hydroxy-ethyl lead to significant activity reduction.

In 1996, the Caliendo group, through binding assays with radiolabeled ligand assays, noted the affinity of previously synthesized derivatives for the following recombinant human receptor subtypes: 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-$HT_{1D\beta}$ [208]. The experimental results shown that most of the compounds possess an high affinity for the 5-HT_{2A} receptor, while none of them bear affinity for 5-$HT_{1D\beta}$ receptor. The bond to the 5-HT_{2A} receptor is favored by groups Ph, 3-Cl-Ph or 4-Cl-Ph on the N-4 piperazine ring. Instead, as regards to the 5-HT_{1A} receptor, the potency increase significantly when a substituent 2-MeO-Ph is located at the N-4 piperazine ring. Then the receptors binding affinity is due to the double effect of the R substituent and the type of carbon linker. This means that there are different modes of interaction between ligand and receptor, as postulated by Ismaiel *et al*. [156] The research group of Caliendo has also compared the binding affinity and selectivity of the derivatives against different receptor isoforms, expressing it as 5-HT_{2A}/5-HT_{2C} ratio. The results of the experiments demonstrate that the best selectivity ratio is obtained when R is a Ph or 4-Cl-Ph group. On the contrary, when R is represented by a 2-MeOPh group is obtained a receptor ligand that acts as an antagonist toward the 5-HT_{1A} receptor.

Other potential receptor ligands and trazodone like compounds are those reported by Sparatore *et al.*, whom analyzed for many years the benzotriazole pharmaceutical properties. They reported a series of 2-[4-[3-(4-aryl/heteroaryl-1-piperazinyl)propoxy]phenyl]- 2*H*-benzotriazoles and their N-oxides [209], which design is based on previous biological evidence reported for a series of 2-4-(dialkylaminoalkoxy)phenyl]benzotriazoles and corresponding N-oxides [197]. Fig. 33 shows the above series.

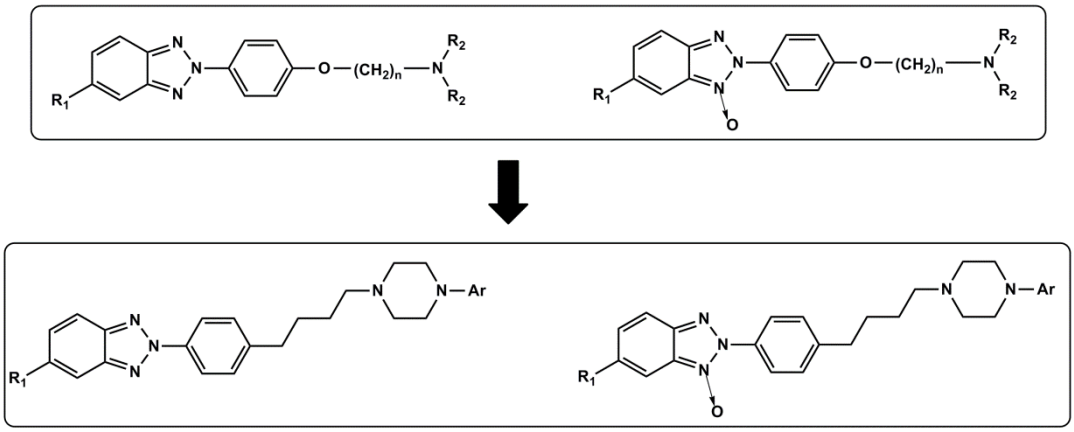


Fig. 33. Piperazinyl-phenyl-2*H*-benzotriazoles derivatives and their N-oxides.

Indeed, the aryl/heteroaryl moiety 3-trifluoromethylphenyl is a well known ligand for 5-HT1 and 5-HT2 receptors , but the affinity of these derivatives for dopamine and adrenergic α_1 receptors has not been excluded [210, 211]. The synthesized compounds were able to bind dopamine receptors D_2 and D_3 and serotonin receptors 5-HT_{1A} and 5-HT_{2A}, but they present low affinity for subtypes 5-HT_{1B}, 5-HT_{2C}, 5-HT_3, and 5-HT_4.

Also the next 2-(4-[ω-[4-(2-Methoxyphenyl)-1-piperazinyl]alkoxy]phenyl)-2*H*-benzotriazoles and their N-Oxides shown an affinity for 5-HT_{1A} and D3 receptors, affinity that increases with the length of the aliphatic linker [212]. Even if the benzotriazole nucleus is identified as provider to the affinity towards the 5-HT_{1A} and 5-HT_2 receptors, the 4-[3-(benzotriazol-1-yl)propyl]-1-(2-methoxyphenyl)piperazine was instead identified as potent receptor antagonist for the pre-and post-synaptic 5-HT_{1A} receptors [213].

Paluchowska and coworkers highlighted the relevance of the aliphatic linker length between methoxy phenyl piperazines moiety and the benzotriazole nucleus. They synthesized and evaluated a series of aryl piperazine in which the linker was composed of two to four methylene groups. Part of this set has proved to act as a ligand of the 5-HT_{1A} receptor, with Ki between 4 and 88 nM. The derivatives with bi-methylene bridges results *in vivo* inactive, while those with tetra-methylene bridges proved to operate as antagonists for postsynaptic 5-HT_{1A} receptors [214].

More aryl/heteroaryl-piperazinyl alkyl benzotriazoles were prepared and evaluated, by modification of the benzotriazole moiety (introduction of substituents such as Cl and OCH_3) or the aryl side chain (introduction of 2-pyrimidinyl or 3-trifluoromethylphenyl groups). These modifications have been conducted in an attempt to increase the affinity of the derivatives to the 5-HT_{1A} receptors, and new compounds have in fact demonstrated moderate to good affinity towards serotonin receptor, but none or modest affinity for dopamine D2 receptor [215].

Finally, demonstrating the significance of the structures previously reported, here is reported the

failure of the derivatives trifluoromethyl and methoxyphenyl piperazin-1-yl-ethoxy-1*H*-benzotriazoles, described in Fig. 34, that proved to be completely inactive as $5HT_{1A}$ and D2 competitor [216].

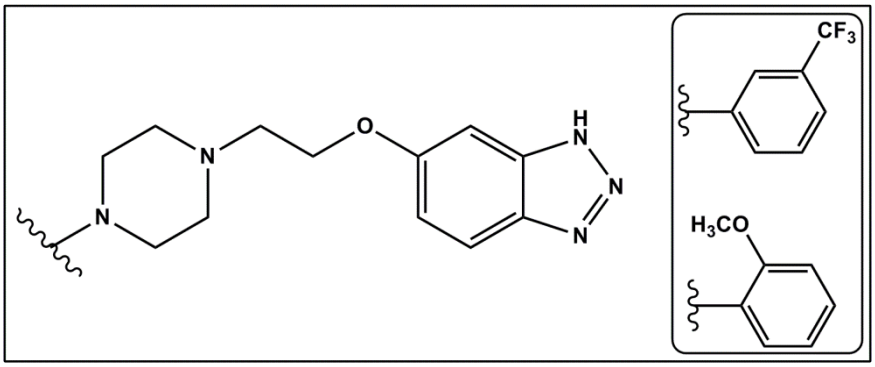


Fig. 34. 6-(2-(4-Methylpiperazin-1-yl)ethoxy)-1*H*-benzo[*d*][1,2,3]triazole derivatives.

CHOLERETIC, CHOLESTEROL-LOWERING AND ANTI-INFLAMMATORY ACTIVITY OF BENZOTRIAZOLE

In 1964 Sparatore group described a series of benzotriazolylalkanoic acids, including the 3-(benzotriazol-l-yl)butanoic acid, which structure is detectable in Fig. 35, emerging for its strong choleretic activity [217, 218]. On this model was therefore carried out a series of structural changes in order to understand the meaning of modifications on the aromatic moiety of the heterocycle [219, 220]. Results demonstrate that the introduction of a methyl or a methoxy group at 6 position of the benzotriazole ring cause a decrease in biological activity compared to the 3-(benzotriazol-l-yl)butanoic acid, while the presence of a trifluoromethyl group determines its increase.

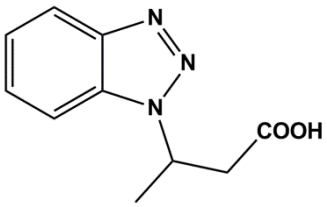


Fig. 35. 3-(Benzotriazol-l-yl)butanoic acid.

Also the modification of the side chain on the benzotriazole nitrogen N-1 has consequences: the activity is maintained *introducing unsaturation* into the N-alkyl chain, but is lost *shortening* it [221]. For some of these derivatives the antinociceptive and anti-inflammatory activity was also evaluated [222].

Researcher synthesized so many other alkanoyl acids, including a series of 4'-(benzotriazol-2-yl-phenylalkanoic and -phenoxyalkanoic acids their N-oxides with different biological properties, such as anti-inflammatory, diuretic or anti-hypertensive [223, 224].

Over the years additional modification have been done basing on Buu-Hoi studies [225], which determined, for some substituted benzimidazoles such as 2-[4-(2-diethylaminoetoxy)phenyl]benzimidazole, strongly depressive effects on the CNS and inhibitor activity against cholesterol biosynthesis.

Basing on this evidences a series of 2-[4-(dialkylaminoalkoxy)phenyl]benzotriazoles and their N-oxides has been synthesized [197], as described in Fig. 36:

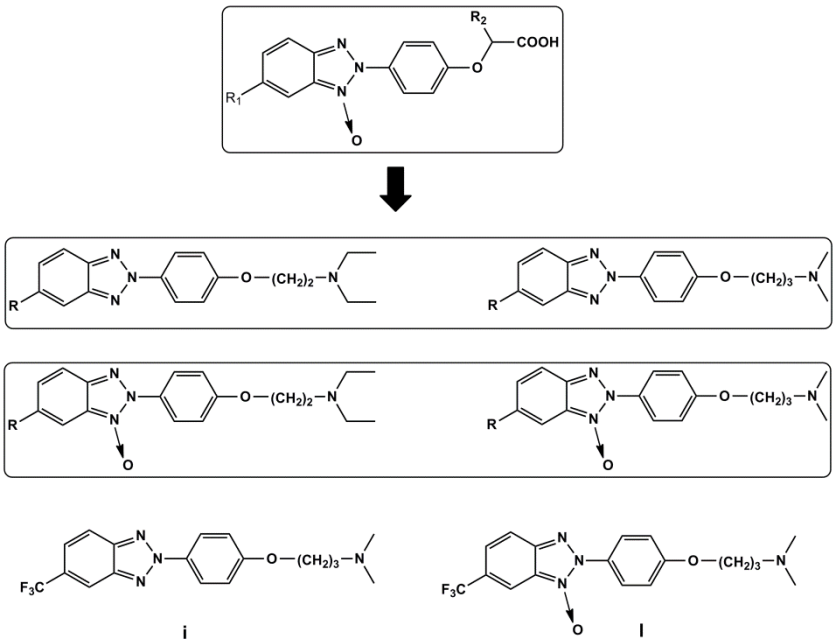


Fig. 36. Series of 4'-(benzotriazol-2-yl-phenylalkanoic and -phenoxyalkanoic acids and derivatives.

The pharmacological investigation demonstrated that compounds **i** and **l** showed different behavior:

1) to prevent the death by hypoxia in mice treated with potassium cyanide. This effect is normally exercised by calcium antagonists, such as cinnarizine and flunarizine. This effect may be related to antagonism versus leukotriene D4 receptor, effect exhibited by both derivatives;
2) the inhibition of thromboxane A2, exerted by compound **i**. The latter showed also anti-atherogenic properties.
3) the diuretic action exerted by the compound **l**.
4) strongly cholesterol-lowering activity of both derivatives.

Widely used to synthesize new compounds of pharmaceutical interest, benzotriazole, as mentioned so far, stands out for its multiple biological activities, including the anti-inflammatory, anesthetic and anticonvulsant. 1-lupinylbenzotriazoles have been tested for anti-inflammatory, diuretic, analgesic and anti-hypertensive activity, obtaining good results in one or more areas [226]. Instead, compounds *N*-[2-(tert-amino)ethyl]- and *N*-(lupinyl)-benzotriazol-1/2-yl acetamides, assessed as local anesthetics, have shown an activity comparable or higher to that of lidocaine [227].

The benzotriazole ring was also combined with tetrazole systems in order to enhance the antinociceptive and anti-inflammatory properties of both nuclei [228]. Among the reported in the scientific literature 1-[2-(1*H*-tetrazol-5-yl)ethyl]-1*H*-benzo[*d*][1,2,3]triazoles derivatives, the 5-(2-(1*H*-benzo[*d*][1,2,3]triazo-1-yl)ethyl)-1*H*-tetrazol-1-yl)(4-aminophenyl)methanone and (2-hydroxyphenyl)methanone emerge for antinociceptive properties, being able to triple and quadruple, respectively, the reaction time in the hot plate test. Instead, the 1-(2-(1-tosyl-1*H*-tetrazol-5-yl) ethyl)-1*H*-benzo[*d*][1,2,3]triazole and 4,5-(2-(1*H*-benzo[*d*][1,2,3]triazo-1-yl)ethyl)-1*H*-tetrazol-1-yl)sulfonyl)benzenamine possess anti-inflammatory activity superior to that of the derivatives of the same series (Fig. 37).

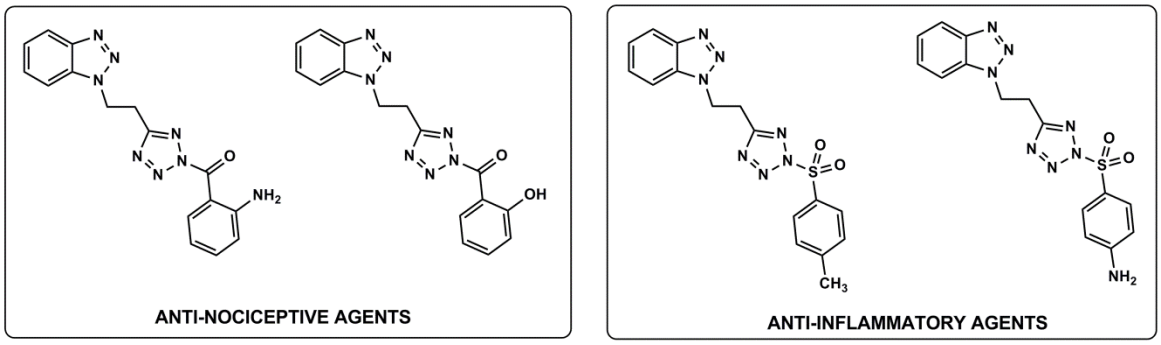


Fig. 37. Tetrazolyl-benzotriazole derivatives endowed with anti-nociceptive and anti-inflammatory activity.

Among the new derivatives that can fit in the category of non-steroidal anti-inflammatory agents there is to count a number of compounds obtained by the combination of both the benzotriazole and benzofuranic nuclei [229]. These derivatives were evaluated concerning the antinociceptive and anti-inflammatory activities. The 3-acetyl-4-(1-benzotriazolyl)-5-(2-benzofuryl)-1-(*p*-chlorophenyl)pyrazole stands out for its analgesic action, and SAR studies show that the pyrazole nucleus is more effective than the 1,3,4-thiadiazole ring. The results obtained in several animal models suggest that these derivatives exert centrally and peripherally mediated antinociceptive properties. Almost all of the prepared compounds also expressed anti-inflammatory activity, with a maximum for the 2-(5-acetyl-3-phenyl-1,3,4-thiadiazol-2-ylidene)-1-(2-benzofuryl)-2-(1-benzotriazolyl)-ethanone. SAR analysis of 5-acetyl-1,3,4- thiadiazole derivatives demonstrates that such activity decreases with the modification on the phenyl side chain. Studies on the mechanism of action have finally shown that new compounds act through a selective inhibition of COX-2 [230].

BENZOTRIAZOLE AS ANTIVIRAL AGENT

Viruses are pathogens agents that can cause major diseases both in human and animals, determining life lost, economical loss and higher productivity costs. Bartorfd classification part them in DNA

and RNA viruses, tabulation genomic based. Numerous targets can be used to develop antiviral agents, and a large part of them is represented by enzymes, such as polymerase, protease, and helicase. Nucleosides and non-nucleosides inhibitors are widely investigated to implement the pharmacological arsenal and to obtain more potent and selective antiviral agents. Resulting from the potent bioactivity of benzotriazole, several derivatives act as antiviral agents, objecting to a variety of targets and showing sometimes an interesting selectivity of action.

Starting from helicases, synthesis and biological activity of benzotriazole analogous as NTPase/helicase inhibitors has been extensively investigated [231]. As recently reported NTPase/helicase is a promising target and ssRNA+ (positive sense single-stranded RNA) enzymes have been studied in detail [232], since ssRNA+ viruses, belonging to families like Flaviviridae and Picornaviridae, continues to pose threats to public health [233, 234]. Benzotriazole helicase inhibitors represent an interesting class of drugs, and potent derivatives were identified during the course of random screening studies. Particularly, previously reported as CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBBt), and 5,6-dichloro-1-(β-D-ribofuranosyl) benzotriazole (DRBT) displayed an antiviral activity, showing IC_{50} values of 20 and 1.5 μM, respectively [23]. DRBT and TBBt have been tested in four different HCV subgenomic replicon systems, resulting both able to inhibit HCV replication ($EC_{50\ DRBT}$ =10–53 μM, $EC_{50\ TBBt}$ = 40–65 μM) in a comparable way to the inhibition reported in the enzymatic essays, showing a property that has been detected only for a handful group of HCV inhibitors. Neither they are cytotoxic at concentrations up to 100 μM [235]. Furthermore, N-alkyl derivatives of TBBt showed good inhibitory activity against HCV, WNV, and JEV NTPase/helicases, and less cytotoxicity [236]. The relevance of the benzotriazole moiety was finally demonstrated by replacing it with a benzimidazole in the 5,6-dichloro and 5,6-dibromo-1-(β-D-ribofuranosyl) benzimidazole (DRB and DBRB), much less potent in inhibit HCV helicases (Fig. 38).

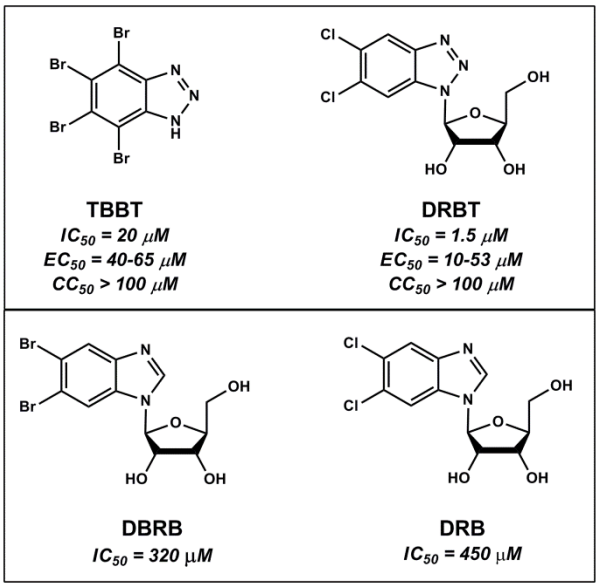


Fig. 38. Structures and anti HCV (DNA substrate) activity of benzimidazole and benzotriazole derivatives.

To explain this finding, Bretner *et al.* synthesized and studied a new series of N-substituted 1*H*-benzimidazole and 1*H*-benzotriazole [231]. The starting compounds 1*H*-benzotriazole and 1*H*-benzimidazole showed a very low anti HCV-helicase activity on a DNA substrate (IC_{50} 200 μM and 500 μM, respectively) and no activity (IC_{50} > 500 μM) when measured either with an RNA substrate or against the enzymes of WNV, DENV, and JEV (family: Flaviviridae). On the contrary, the whole brominated 1*H*-benzotriazole revealed to be 9-10 fold more effective in inhibit the HCV helicase when determined with a RNA or DNA substrate (more potency in case of JEV helicase, IC_{50} 20 μM). Moreover, the brominated 1*H*-benzimidazole resulted to be less effective than TBBt but more potent than the non-halogenated parent compounds against HCV helicase. To enhanced hydrophobicity, N-alkylated derivatives (substituted with methyl, ethyl and propyl moieties) were synthesized, as shown in Fig. 39.

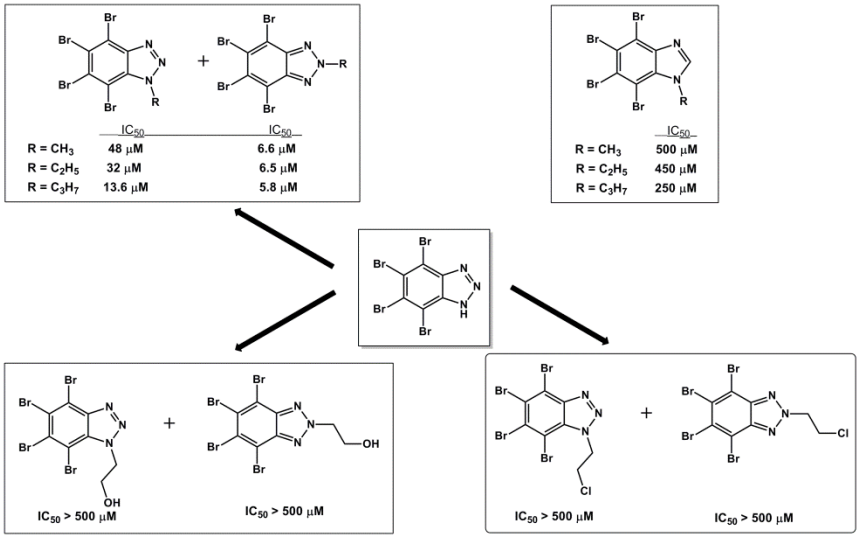
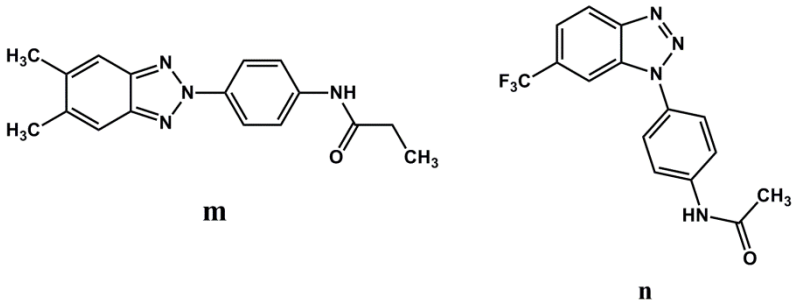


Fig. 39. Structures and anti-HCV helicase activities of 1*H*- tetrabromobenzotriazole and tetrabromobenzimidazole derivatives using a DNA substrate.

The comparison of 1- or 2-alkyl benzotriazoles effectiveness on the HCV-helicase using the DNA substrate demonsrated that the 2-alkylated derivatives resulted significantly more potent as helicase inhibitors, while in 1-alkylated benzotriazoles and benzimidazoles the aliphatic chain elongation determined an activity enhancement. However, in DNA substrate the benzimidazole derivatives inhibitory activity was very low (ranged between 250 and 500 μM), and no inhibitory activity was observed using the RNA substrate, as well as using other viral NTPase/helicases. This behavior suggests that these inhibitors do not act through a block on the enzymatic NTP binding sites, and that the occupation of an allosteric nucleoside binding site should be considered, as previously suggested by Porter [237].

Investigating the role of hydrophobic N-alkyl substituents, the authors observed that replacement of the alkyl side-chain by a substituent endowed with higher hydrophilicity (such as in hydroxyethyl derivatives) or with higher hydrophobicity (such as in chloroethyl derivatives) severely decreases the activity of the TBBt derivatives, demonstrating that a small hydrophobic alkyl moiety (methyl or ethyl) at TBBt position 2 could play a crucial role in the inhibition of HCV NTPase/helicase. Finally, authors report that introduction of a ribofuranosyl ring in both benzotriazole and tetrabromobenzotriazole improves the water solubility but leads to a decrease of the inhibitory activity against HCV and all the enzymes tested [23].

More NS3-targeting benzotriazole were investigate by Carta and coworkers, that evaluated *in vitro* cytotoxicity and antiviral activity against a wide spectrum of ssRNA+ viruses of a series of *N*-[4-(1*H*(2*H*)-benzotriazol-1(2)-yl)phenyl]alkylcarboxamides [238]. From this study emerged that the Enteroviruses CVB-2 and Sb-1 (Picornaviridae family) were the only inhibited by title compounds. This is an interesting target, since actually no specific antiviral therapy is available for the treatment of Picornaviridae infections. Particularly, two of them emerged for their selectivity: N-(4-(5,6-dimethyl-2*H*-benzo[*d*][1,2,3]triazol-2-yl)phenyl)acetamide (**m**), which was the most active against CVB-2 (EC_{50}=10 μM and CC_{50}>100 μM) and N-(4-(6-(trifluoromethyl)-1*H*-benzo[*d*][1,2,3]triazol-1-yl)phenyl)acetamide (**n),** which was the most active against Sb-1 (EC_{50} = 30 μM and CC_{50}=90 μM) (Fig. 40).



m **n**

Fig. 40. N-[4-(1*H*(2*H*)-benzotriazol-1(2)-yl)phenyl]alkylcarboxamides derivatives.

SAR analysis suggest that the potency and selectivity of **m** is probably due to the small electrondonor methyl group at positions 5 and 6, a phenyl group at position 2 and a propanoyl-amide group at 4′, while the selectivity of **n** is correlated to a CF_3 group at position 6, a phenyl at position 1 and an amide group at 4′. Title compounds were also evaluated *in silico* against the Polio virus (Sb-1) helicase, which 3D model was obtained by homology techniques. Molecular dynamics simulations showed that all inhibitors are able to rank binding affinities with a similar docking mode in the putative binding site, as depicted in Fig. 41.

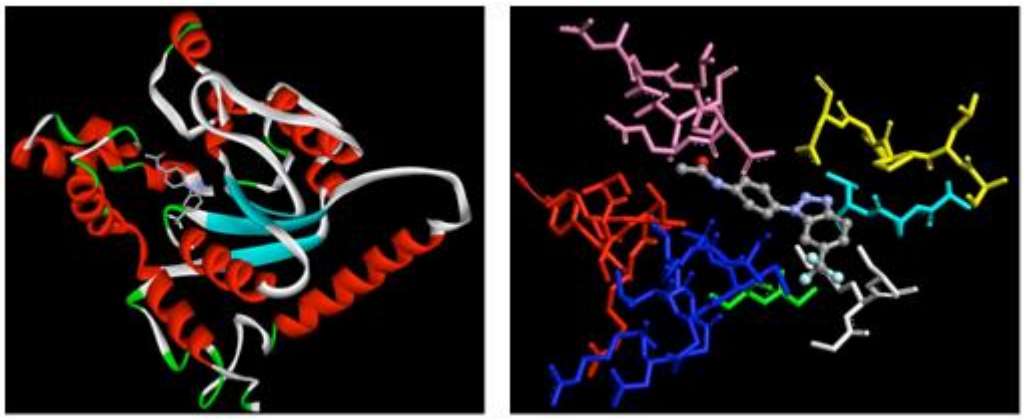
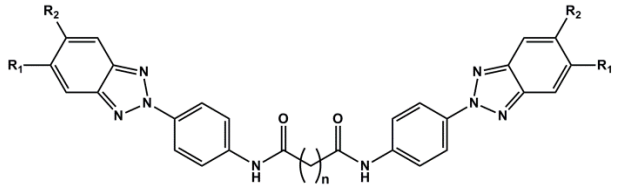


Fig. 41. Cartoon of the binding mode of compound **n** in the putative binding site of the Polio (Sb-1) helicase and snapshot of the docked compound in the putative binding site.

Using these molecules as lead compounds and making use of Diana's patent concerning N,N'-bis[4-(2-benzimisazolyl)phenil]alkyldicarboxamides [239], Carta *et al.* prepared a series of *N*,*N*-bis-[4-(1*H*(2*H*)-benzotriazol-1(2)-yl)phenyl]alkyldicarboxamides [240]. Evaluated in parallel cell-based assays for cytotoxicity and antiviral activity, none of the them resulted active against representative RNA viruses, while best activity is reported for (2*H*) analogues. Among them bis-5,6-dimethyl-derivatives (**1-3**) exhibited good activity against Enteroviruses (EC_{50} = 7–11 μM against CVB-2, EC_{50} = 19–52 μM against Sb-1), and some bis-5,6-dichloro-benzotriazol-2-yl derivatives (**4-6**) emerged for the interesting selective activity against CVB-2 (EC_{50} = 4–11 μM) resulting to be completely inactive against all the other viruses screened, pairing this behavior with a good no cytotoxic profile (Fig. 42).



	$R_1 = R_2$	n	CC_{50} MT-4	EC_{50} CVB-2	EC_{50} Sb-1
1)	CH_3	2	> 100 µM	11 µM	52 µM
2)	CH_3	3	> 100 µM	8 µM	43 µM
3)	CH_3	4	42 µM	7 µM	19 µM
4)	Cl	2	> 100 µM	4 µM	> 100 µM
5)	Cl	3	> 100 µM	11 µM	> 100 µM
6)	Cl	4	> 100 µM	9 µM	> 100 µM

Fig. 42. *N*,*N*-bis[4-(2*H*)-benzotriazol-(2)-yl)phenyl]alkylcarboxamides and their antiviral activities.

Finally, an extended binding mode study was performed on Sb-1 helicase previously obtained through homology techniques. Furthermore, in the absence of a 3D model for the CVB-2 helicase, the activity of **4-6** derivatives was explained adopting a 2D alignment analysis.

Benzotriazole derivatives were also found to be very interesting protease inhibitors toward a new breaking out human Coronavirus: the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) [241]. Spreading from southern China in late 2002, in 2003 SARS became epidemic, rapidly *diffonded* from its origin to more than 25 countries. It affected almost 8000 patients and cause the death of about 10% of those infected, a high mortality rate [242].

Since no effective anticoronaviral therapeutics are available, considering the lingering danger to human health represented by SARS-CoV as well as other coronaviruses, a search for helpful antivirals for the SARS-CoV is of current interest. The most important enzymatic target that the scientifically community identified is represented by a protease, the main proteinase or M^{pro} (also called dimeric chymotrypsin-like protease or $3CLpro^{pro}$) [243], that cleavage two polyproteins, pp1a and pp1ab [244, 245], to provide the functional proteins for viral propagation. Only few M^{pro} inhibitors have been reported, like aryl boronic acid [246], keto-glutamine analogues [247], phtalhydrazide ketones [248], an α,β-epoxyketone [249], thiopurine analogues [250].

In 2006, Wu *et al.* reported a new class of stable benzotriazole esters, which appeared to act as $3CL^{pro}$ irreversible inactivators, with inhibition constants in the nanomolar range [251]. All derivatives were obtained using the strategy of combinatorial reaction in microtiter plates followed by screening *in situ* [250-252]. Particularly, benzotriazole esters were prepared by condensation of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) with 90 various carboxylic acids. Only the derivatives that resulted stable at pH 5.0–8.0 over 24 hr at room temperature were screened. All compound are not cytotoxic (CC_{50} on Vero E6 cells > 100 µM), and the 1*H*-indole-5-carboxylic acid benzotriazol-1-yl ester (Fig. 43) stood out, exhibiting a k_{inact} of 0.0011 s^{-1} and a K_i of 7.5 nM.

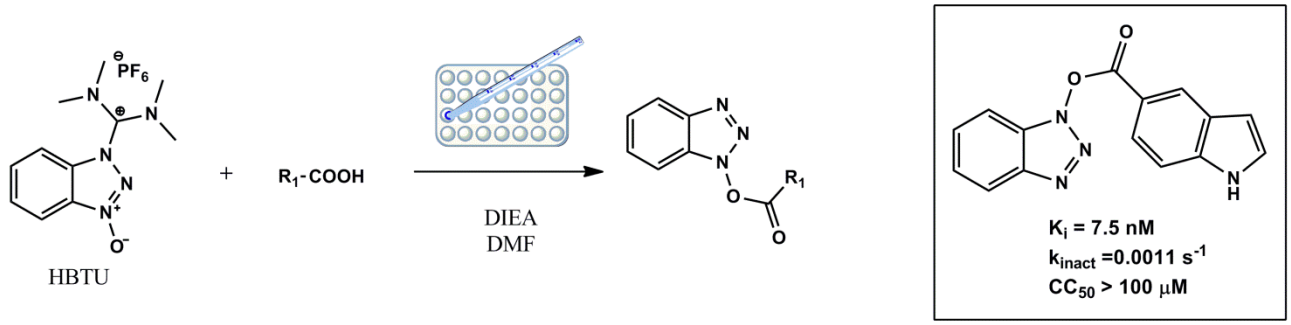


Fig. 43. Synthesis of benzotriazole esters starting from HBTU and 90 acids in microtiter plates, and 1*H*-benzo[*d*][1,2,3]triazol-1-yl 1*H*-indole-5-carboxylate activity.

An extensive mode of action study suggest that these benzotriazole esters could determine inhibition of 3CLpro^{pro} *via* irreversible acylation of Cys145, as shown in Fig. 44. Particularly, Cys145 and His41 are a essential at the protease active site. They act as the principal catalytic residues, in which the cysteine thiol functions operate as nucleophile in the proteolytic process [252]. However, even though the registered Ki values are among the lowest for any inhibitor of the SARS-CoV 3CLpro^{pro} described so far, authors also reported that full inhibition of the enzyme could not be achieved.

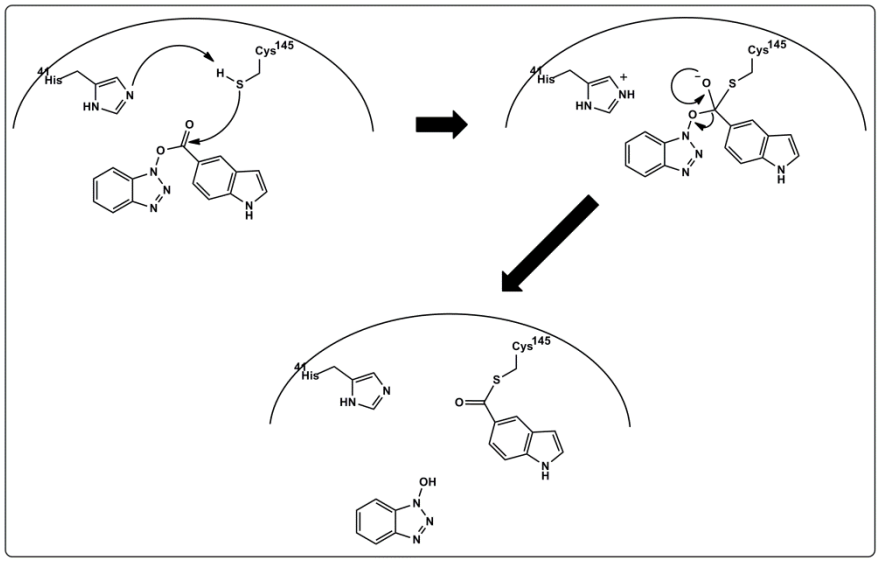


Fig. 44. Proposed mechanism for inhibition of SARS-CoV 3CLpro^{pro} by acylation with benzotriazole esters by Wu *et al.*

Finally, additional benzotriazole esters proposed in the paper were developed to obtain more stable derivatives, in which ester oxygen is replaced by a carbon. Unfortunately they resulted weaker than the corresponding esters, and they bind non-covalently to the enzyme.

Verschueren *et al.* studied the binding mode of benzotriazole esters to the SARS-CoV main proteinase using two of such compounds, 1-(benzoyloxy)-benzotriazole (compound XP-27) and 1-(4-dimethylaminobenzoyloxy)-benzotriazole (compound XP-59) [253]. These derivatives are able

to inhibit the enzymatic activity at low micromolar concentrations (<5 μM and 0.1 μM for XP-27 and XP-59, respectively). Authors demonstrate that benzotriazole esters act as suicide inhibitors by covalently binding to the proteinase.

So far, benzotriazole derivatives where individuate as ssRNA+ viruses inhibitors. However, almost one Single-stranded negative strand RNA virus was identified as a target for compounds bearing a benzotriazole moiety: the Human Respiratory Syncytial Virus(RSV), classified in the genus Pneumovirus of the family Paramyxoviridae. RSV is the leading cause of acute respiratory tract infections in persons of all ages but can also cause serious lower respiratory tract infections in infants aged < 6 months, infants born before 35 weeks of gestation and infants and children with underlying lung disease or congenital heart disease [254], elderly and immunocompromised persons. Severe infection of the virus may result in bronchiolitis or pneumonia which may require hospitalization or result in death. The World Health Organization estimates that RSV causes 64 million infections and 160,000 deaths annually [74]. Currently only Ribavirin is approved for the treatment of this viral infection, as for some others emerging/neglected viral infections, and its efficacy has remained controversial. Therefore, is still mandatory to identifier effective therapeutic options that can treat RSV infections in the at-risk population.

In this perspective is collocate the paper by Yu et coworkers. In 2003 the group reported 1-[(dialkylamino)alkyl]-2-[(benzotriazol-1/2-yl)methyl] benzimidazoles as a new class of inhibitors able to protect HEp-2 human lung carcinoma cell line cells against *RSV cytopathic effects* [255]. Indeed, these compounds were protected by a Bristol-Myers Squibb Company patent [256], but a part of them were first disclosed by Pagani [217] and Paglietti [257], which found them to posses analgesic and antiarythmic activity. Lead compounds of this first series of new RSV-inhibitors are depicted in Fig. 45. Particularly, 2-(2-((2*H*-benzo[*d*][1,2,3]triazol-2-yl)methyl)-1*H*-benzo[*d*]imidazol-1-yl)-N,N-diethylethanamine demonstrate potent antiviral activity against both A and B subtypes of RSV with a good cytotoxicity profile paired with EC_{50} values an order of magnitude lower than that of ribavirine (EC_{50} = 2.7, CC_{50} = 34 μM, data not shown) [258].

Analysis of structure-activity relationships centered to the variation of the dialkylaminoalkyl side chain determined a wide tolerance to structural variation, and both polar and lipophilic functionality at the chain terminus preserve the RSV inhibition. However, the only requirement is a minimum of two atoms of separation between terminus and the heterocyclic moiety. Authors also established that the topological relationship of benzotriazole respect the substituted benzoimidazole moiety is not critical since both isomers proprieties were essentially equal [255, 259].

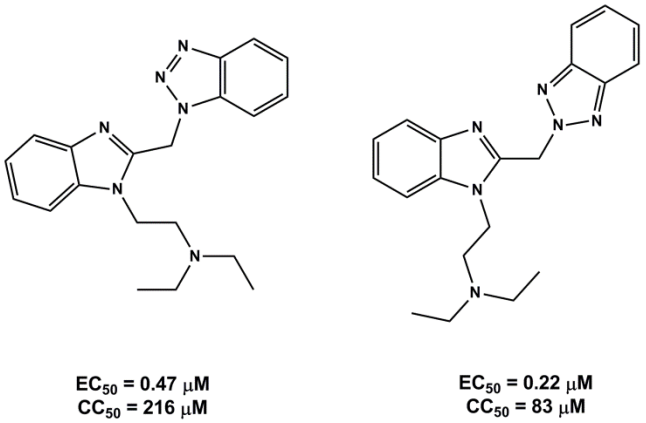


Fig. 45. Lead compounds benzotriazole-derived that act as inhibitors of Respiratory Syncytial Virus fusion.

Unfortunately for our review, the broad survey designed to delineate the pharmacophore associated with leads compounds guided Yu *et al.* to replace the benzotriazole element with a benzimidazol-2-one moiety. This modification led to even more potent anti-RSV agents and to broad tolerance for functionality in this region of the pharmacophore [260].

However, Tonelli *et al.* extended the analysis on 1-Substituted-2-[(Benzotriazol-1/2-yl)methyl]benzimidazoles following the report from Bristol researchers. They evaluated the antiviral activity of related 1-substituted 2-[(benzotriazol-1/2-yl)methyl]benzimidazoles, firstly prepared by the group and not examined by Yu *et al.* In particular, some 5-substituted derivatives, as well as compounds bearing at position 1 the simple (dialkylamino)alkyl chains or the bulky, strongly basic and lipophilic (quinolizidinyl)alkyl nucleus (Lupinyl, Epilupinyl and Homolupinyl), were re- or synthesized. Derivatives are depicted in Fig. 46.

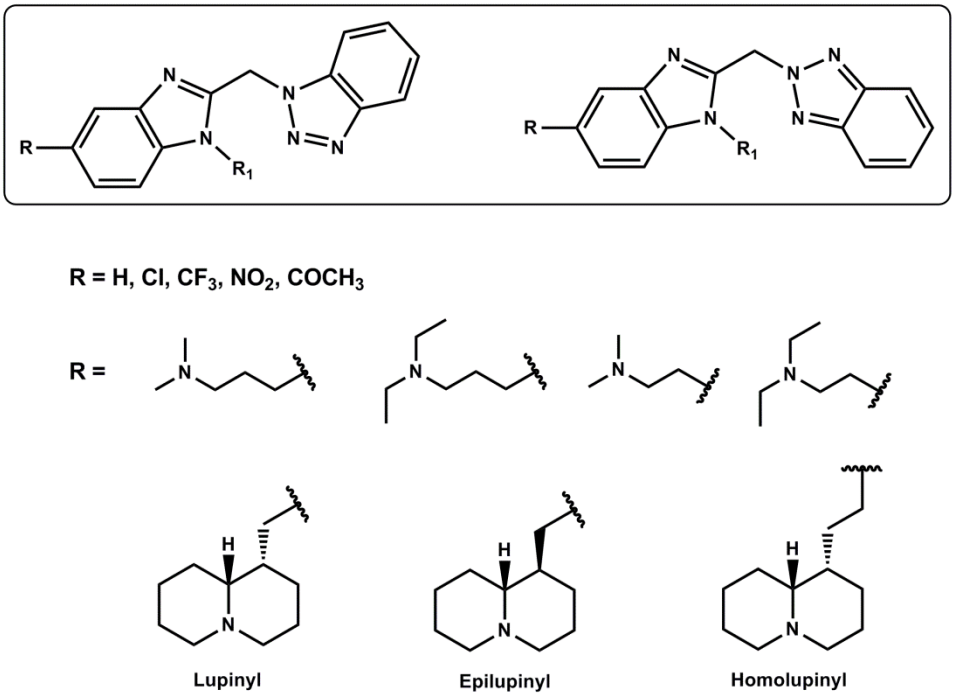


Fig. 46. 1-Substituted-2-[(Benzotriazol-1/2-yl)methyl]benzimidazoles proposed by Tonelli *et al*.

A total of forty-three 1-substituted 2-[(benzotriazol-1/2-yl)methyl]benzimidazoles have been tested for cytotoxicity and antiviral activity against a large panel of RNA and DNA viruses and, among them, thirty-nine compounds exhibited potent activity against RSV, in some cases with EC_{50} values below 50 nM [261]. SAR studies suggested that the presence of substituents at position 5 of the benzimidazole ring leads to an increase in cytotoxicity, especially if compound bear the (quinolizidinyl)alkyl nucleus. Toward antiviral activity, the most potent compounds present a Cl in position 5 paired with an R_1 equal to $(CH_2)_2N(CH_3)_2$ or $(CH_2)_3N(CH_3)_2$, while the replacement of Cl with H, NO_2, CF_3 or $COCH_3$ leads to lower potency. Surprisely, the introduction at R_1 of bulkier group leads to a progressive increase of potency, as seen when $(CH_2)_2N(CH_3)_2$ is replaced with $(CH_2)_2N(CH_2CH_3)_2$ or lupinyl group, and homolupinyl derivates are the most potent, regardless of whether R is H or Cl. Finally, comparison of the two isomeric series, N(2)-substituted benzotriazoles were generally less active.

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