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## Review article

## Benzotriazole: An overview on its versatile biological behavior



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## ABSTRACT

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

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## 1. Introduction

Due to their properties and applications, benzo-fused azoles are a class of heterocyclic compounds of great interest in the pharmaceutical chemistry area. Benzimidazole and its derivatives have been studied for decades [1] and drugs having this heterocycle moiety as main element have been widely used in clinic, for instance as anthelmintic in humans [2].

Benzo-condensed azole containing three heteroatoms, such as bezoxadiazole, benzothiazole and benzotriazole [3,4], have been extensively studied for their broad range of biological activity. However, few reviews were focalized on a single nucleus. Indeed, the aim of this paper is to provide an overview of the benzotriazole based systems and their relevance in medicinal chemistry.

The 1*H*-benzo[*d*][1,2,3]triazole (BT) (Fig. 1) can be considered as a privileged structure for its several pharmacological activities. Useful as scaffold for the design of new pharmacologically active compounds, BT is undergoing rapid development in the synthesis of heterocycles.

From a purely chemical point of view, the benzotriazole structure proved extremely versatile applicabilities. For instance, it is currently used as a synthetic auxiliary [5–10] or as a good leaving group after reaction with a variety of carbonyl groups [10–13]. In particular, it is interesting the use of the acylbenzotriazole

methodology, developed by Katritzky and co-workers [14]. The *N*-acylbenzotriazole is an easy-to-handle acylating agent for advantageous *N*-, *O*-, *C*- and *S*-acylations. New peptidomimetic macrocycles (Fig. 2) were obtained from dicarboxylic benzotriazole [15] using this methodology.

Benzotriazole also acts as an electron-donor or 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 [16–18]. Some authors have also reported the synthesis of stable nitrenium ions using BT as synthon [19]. Polymer-supported benzotriazoles were also used as catalysts for the generation of a tetrahydroquinoline library [20].

However, the main interest on BT is focused in the pharmaceutical field, as suitably substituted benzotriazole derivatives can boast the most different biological properties, including plant-growth regulator [21–24], choleric [25], antibacterial [26], antiprotozoal [27], antiviral [28] and antiproliferative [29] activity.

## 2. Benzotriazole as antimicrobial and antiprotozoal agent

The antimicrobial activity of benzotriazole derivatives have been extensively investigated since the late 1980s, and, together with all azolic rings, they become one of the active highlights in recent years [30]. In the first part of the twentieth century, the discovery and development of antibacterial drugs were major scientific achievements. Despite the investments in antimicrobial drugdiscovery, no new drug class has been found in the past 20

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

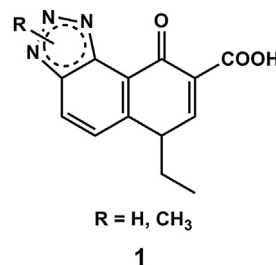


Fig. 3. General formula of triazol[4,5-f]-quinolinone carboxylic acids derivatives.

years [31]. Additionally, the difficulty to treat infections due to antibiotics resistance increase highlighted the urgency to develop new drugs [32]. For some decades, Sparatore and co-workers studied and characterized various nitrogen rings and reported that when BT is part of larger heterocyclic systems, it has biological activities, especially antibacterial [33–35]. In 1989 Sanna's research group also reported the relevance of benzotriazole moiety in triazol[4,5-f]-quinolinone carboxylic acids (**1**) (Fig. 3), closely related to oxolinic acid. These acids showed encouraging *in vitro* antimicrobial activity against *Escherichia coli*, with Minimum Inhibitory Concentration (MIC) values included between 12.5 and 25  $\mu\text{g/ml}$ . Moreover, they demonstrated that annulations in different position of the triazole ring as in triazol[4,5-h]-quinolinone carboxylic acids, induce a partial or total antimicrobial activity loss [36,37].

Derivatives of N-acyl-1H-benzotriazole (**2a**) or N-ethyl-1H-benzotriazole acetate (**2b**) were described by Al-Omran and co-workers, who synthesized a variety of compounds incorporating BT in thiophene, pyridine, thiadiazole or pyrazole moiety (Fig. 4). Most of tested compounds, screened for antimicrobial activity, resulted bactericidal [38]. Potential antibacterial activity was also reported for N-acyl-1H-benzotriazoles substituted with olefinic fatty acids (**3a–d**) (Fig. 4) [39].

Purohit and Srivastava synthesized a series of chlorosubstituted, phenoxyacetyl benzotriazoles (**4a–e**): all compounds, screened for their anti-inflammatory, analgesic, antibacterial and antifungal property, showed mild to moderate antibacterial and antifungal activity. Notably, compound **4c** emerged for its analgesic effect. They also demonstrated that even the simple benzotriazole nucleus possessed antibacterial activity [40]. A similar antimicrobial profile was reported for a series of 1-(1H-benzotriazol-1-yl)-2-(heterocycl)ethanones (**5a–f**) (Fig. 5) [41].

1H-Benzotriazol-1-yl(2-hydroxy-5-[(*E*)phenyldiazenyl]phenyl) methanone derivatives were prepared by Jamkhandi and co-workers through diazonium coupling reaction. These compounds showed from good to moderate antibacterial and antifungal activity. Interesting zone of inhibition diameters were noted for compounds (**6a–b**) in comparison with standards drugs [42]. Same results were also reported for [(1H-benzotriazol-1-yl)acetyl]amino] acetic acid derivatives (**7a–e**) [43], while 2-(1H-1,2,3-Benzotriazol-1-yl)-*N*-phenylacetamide derivatives (**8a–d**) displayed less antibacterial potency [44]. All structures are depicted in Fig. 6.

The *in vitro* antibacterial activity of 5-halogenomethylsulfonyl-benzotriazoles and benzimidazole (**9**) (Fig. 7) were also reported by Ochal's group. All compounds were tested against a series of reference (including Gram-positive and Gram-negative bacteria) and clinical strains (including methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) *Staphylococcus aureus* strains, plus

methicillin-resistant *Staphylococcus epidermidis*). All of them showed significant antibacterial activity, whereas trifluoromethyl-substituent at C-2 benzimidazole derivatives emerged for its potency; indeed, being able to inhibit *Staphylococci* strains (MRSA) with MIC values 12.5–25  $\mu\text{g/ml}$ , they were comparable with nitrofurantoin against some strains [45].

Benzotriazole can also be used to modulate the biological activity of other heterocyclic rings, such as for 9-substituted acridines (**10a,b**) reported by Singh et al. [46], or for levofloxacin derivative (**11**), where 2-aminobenzotriazole is linked to the carboxyl moiety (Fig. 8). In the last case, the modification led to a similar antimicrobial activity as levofloxacin against Gram-positive and Gram-negative bacteria [47].

Das et al. synthesized and evaluated as antimicrobial agents a pool of some novel oxazolidinone derivatives (**12**, Fig. 9). The benzotriazole relevance was confirmed after its replacement with benzimidazole, benzothiazole, or benzoxazole, modifications that provided less active or inactive compounds. Effects of positional and geometrical isomerism on triazole moiety demonstrated that linearly attached derivatives showed higher efficiency compared to angular ones *in vitro* (**12a**). As regards angular derivatives, *E*-isomer (**12c**) was found to be more potent than *Z*-isomer (**12d**). Finally, thioacetamide analogue (**12b**) represented a lead compound endowed with similar activity to linezolid *in vitro* [48].

Besides, a series of novel oxazolidinone derivatives holding (un) substituted-benzotriazoles moieties has been evaluated against a panel of susceptible and resistant Gram-positive and Gram-negative bacteria, some of which resistant to methicillin and vancomycin. The introduction of benzotriazole generated a series of oxazolidinones (Fig. 10) *in vitro* equipotent or much more efficient than linezolid on susceptible and resistant Gram-positive strains. Particularly, the introduction of a –COOMe group in benzotriazole's fifth position led to compounds (**13a**) and (**13b**), which exhibited an excellent antibacterial profile (MIC values of 0.25–0.125  $\mu\text{g/ml}$ ) [49].

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 subtilis*, *Salmonella typhimurium*, *Escherichia coli* and *Bacillus anthracis* at 50 and 100 ppm. Some of them, like compound (**14**) (Fig. 11), resulted to be equipotent to streptomycin [50].

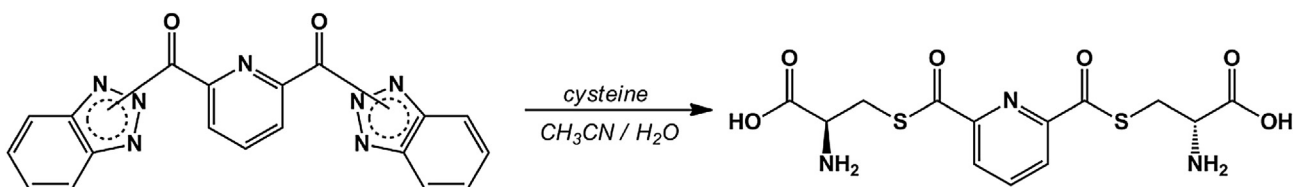


Fig. 2. BT as leaving group.

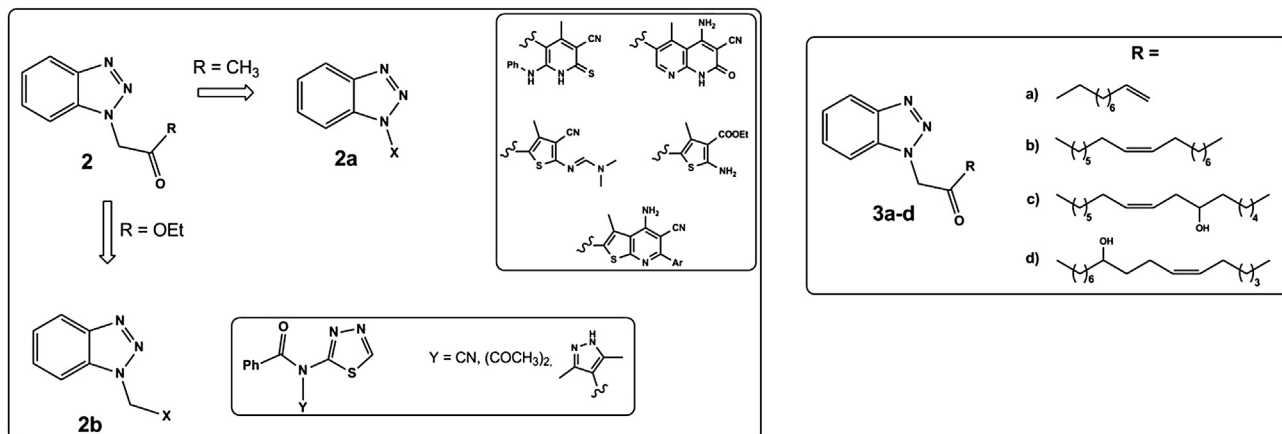


Fig. 4. N-acyl-1H-benzotriazole derivatives endowed with bactericidal activity.

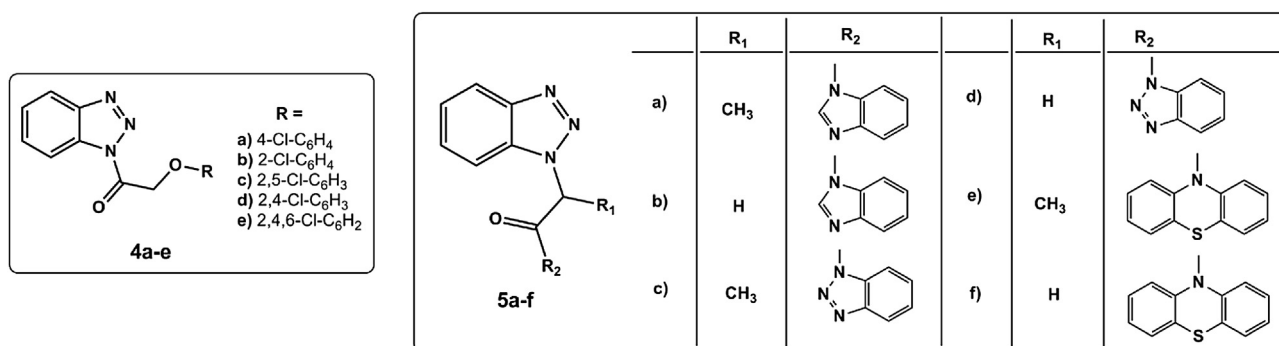


Fig. 5. N-substituted 1H-benzotriazole derivatives endowed with antibacterial activity.

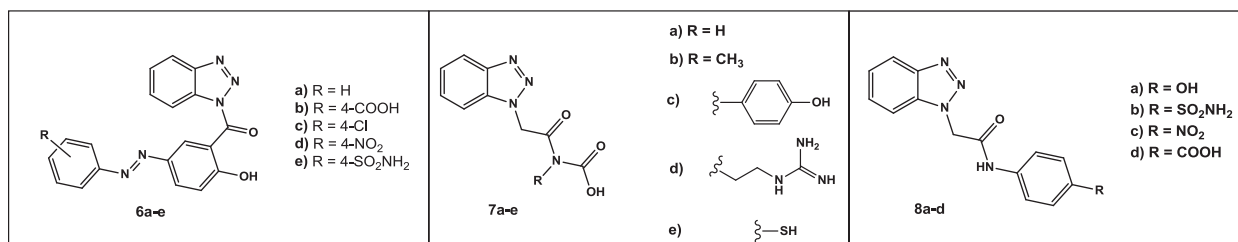


Fig. 6. Chemical structure of diverse benzotriazol-1-ethanone and propan-2-one based derivatives.

In 2006 Swamy et al. prepared a series of N-alkylated benzotriazole derivatives (**15a–g**) through microwave-assisted synthesis [51], as reported in Fig. 12. The antibacterial activity of all compounds was tested against bacterial strains like *Bacillus subtilis*,

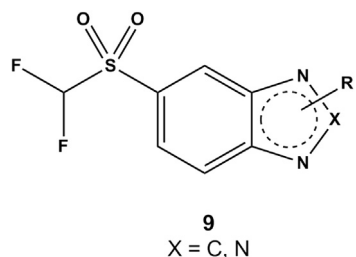


Fig. 7. General formula of difluoromethylsulfonyl-benzotriazole and benzimidazole derivatives.

*Escherichia coli*, *Pseudomonas fluorescens*, *Xanthomonas campestris* e *Xanthomonas oryzae*. Results highlighted how 4'-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-[1,1'-biphenyl]-2-carbonitrile (**15a**) and 1-(6-methylbenzo[d][1,3]dioxol-5-yl)methyl-1H-benzo[d][1,2,3]triazole (**15d**) acted as potent antimicrobial agents. This behavior was probably due to the presence of bulky hydrophobic groups (cyano-biphenyl and benzodioxole). On the contrary, smaller molecules bearing 5-dimethoxy-benzyl (**15e**) and 1-butyl (**15g**) groups resulted equipotent to reference drugs Streptomycin. Finally, even if some derivatives demonstrated to be active on drug-resistant bacterial strains, all compounds lacked of selectivity, being active on both Gram-positive and Gram-negative bacteria over on different fungal strains.

Different authors reported that imidazole and triazole rings are isosteric analogs, synthetically relevant and associated to a variety of biological and pharmacologic activities [52]. Similarly, the N substitution for CH, which transforms benzimidazole in a

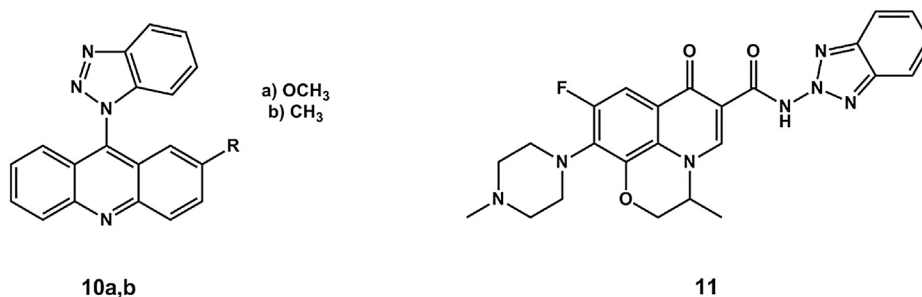


Fig. 8. Benzotriazole nucleus paired with other biologically active heterocyclic rings.

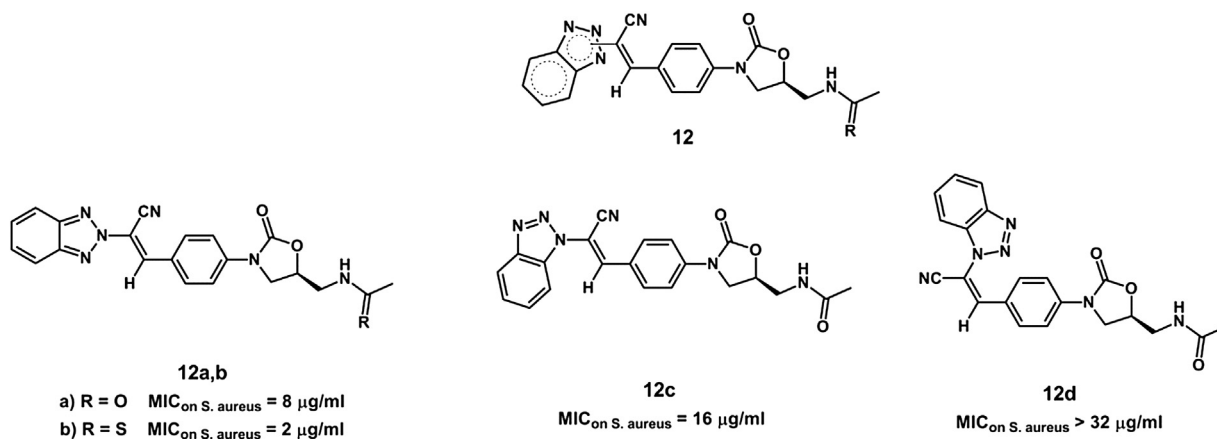


Fig. 9. Oxazolidinones derivatives with benzotriazole as pendant.

benzotriazole derivative, can be considered an example of a classical isosteric replacement [53]. Leading this idea, Ramachandran and colleagues synthesized a series of new imidazole/benzotriazole substituted piperidin-4-one derivatives (**16a–I**, Fig. 13) and evaluated their antibacterial and antifungal activities against some pathogenic microbial strains [54].

This study highlighted how the introduction of a bulky substituent such as an isopropyl group into the piperidine cycle position C-3 increased the antibacterial activity against *Bacillus subtilis* for both imidazole (**16c**) and benzotriazole (**16l**) derivatives (MIC = 12.5 and 6.25 µg/ml, respectively), in comparison to reference drug Streptomycin. A similar behavior was not observed in the antifungal evaluation.

Good growth inhibition against *Escherichia coli* was reported for compounds bearing methyl groups at C-3 and C-5 in the piperidine ring for imidazole derivative (**16d**) (MIC = 6.25 µg/ml). Among the benzotriazole derivatives the best inhibition activity (MIC<sub>on</sub>

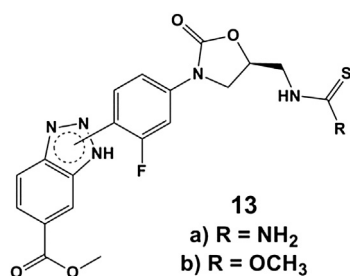


Fig. 10. Benzotriazolyl oxazolidinone derivatives by Dixit et al.

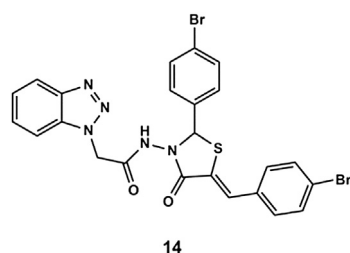


Fig. 11. Chemical structure of hit compound from Asati's work.

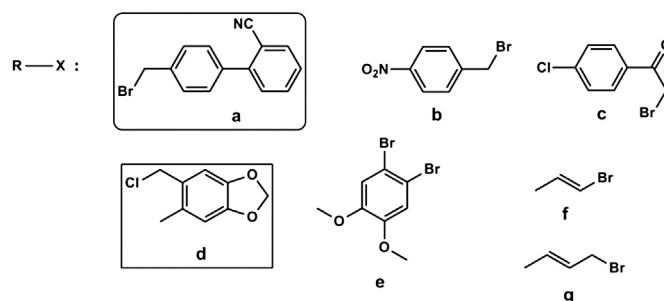
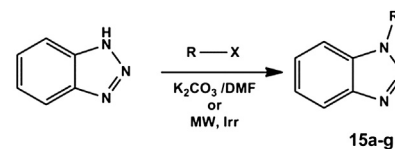


Fig. 12. Microwave-assisted synthesis of N-substituted benzotriazole derivatives.

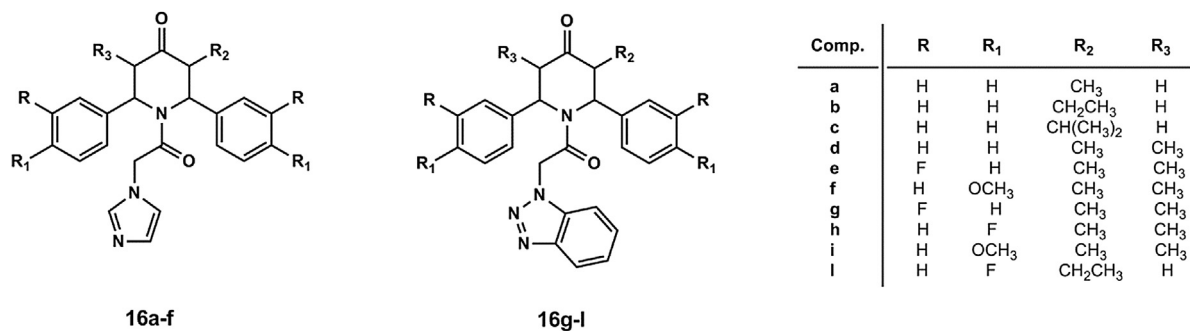


Fig. 13. Imidazole/benzotriazole substituted piperidin-4-one derivatives.

*E. Coli* = 6.25 µg/ml 12.5 mg/mL) was registered for compound (**16h**), containing *p*-fluorophenyl substituent at C-2 and C-6 in the piperidine ring. Most of these compounds showed none or moderate growth inhibition activity on different fungal strains, except for compounds (**16c**) and (**16h**) against *Aspergillus niger* (12.5 µg/mL), (**16e**) against *Candida neoformans* (12.5 µg/mL), (**16f**) against *Candida albicans* (6.25 µg/mL) and (**16h**) against *Rhizopus species* (6.25 µg/mL).

Finally, Suma's research group investigated the antimicrobial properties of a series of (un)substituted-benzotriazoles N-1 linked to substituted pyrazolidin-3,5-dione moiety [55]. All synthesized compounds (**17a–i**) were characterized and biologically evaluated by the cup plate diffusion method. Between the examined derivatives (Fig. 14), only compound (**17h**) was found to be comparable to Ciprofloxacin against *Staphylococcus aureus*, while limited activity is reported against *Candida albicans*. Unfortunately, no SAR analysis was reported.

Solid complexes of ligands containing both the benzotriazole and thioamide moieties with some transition metal ions (**Me**) were investigated as antimicrobial agents. New Cu<sup>2+</sup> and Ni<sup>2+</sup> complexes of N1-phenyl-2-[1*H*-1,2,3-benzotriazol-1-yl]3-phenyl-3-oxopropane thioamide (**18**, Fig. 15) [56] were synthesized and their biological activity evaluated against *Staphylococcus aureus* and *Salmonella* strains. Authors demonstrated that ligand (**18**) acted as bidentate ligand, giving distorted tetragonal structure in case of Cu<sup>2+</sup> complexes and square planar structure in case of Ni<sup>2+</sup>. This difference justified the divergent antimicrobial behavior of derivatives: even if neutral complexes were obtained in both cases, only [L<sub>2</sub>Cu] complex was active against both *S. aureus* and *Salmonella* strains [57].

Finally, little is known about the antiprotozoal activity of benzotriazole derivatives. A simply substitution on BT generated 6-chloro-1*H*-benzotriazole (**19**, Fig. 16), a compound endowed with micromolar activity against *Entamoeba histolytica*. The biological behavior demonstrated that this scaffold, as indazole nucleus, is

more active than metronidazole, the drug most commonly used in the treatment of anaerobic protozoan parasitic infections [27]. Pagliero's works demonstrated that compounds possessing the heterocyclic nucleus 2-methyl-1,2,3,4-tetrahydroquinoline linked to a benzenesulfonyl moiety showed protozoan antiparasitic properties [58,59]. On this basis, through the fragment-based drug design strategy, Becerra's group designed and prepared a library of N1-benzenesulfonyl derivatives of BT [60]. Following the procedure reported by Katrinsky [61], all compounds were prepared by sulfonylation. The group determined the biological *in vitro* activity of N-benzenesulfonylbenzotriazole (**20**, Fig. 16) on the protozoan parasite *Trypanosoma cruzi*. Different concentrations of N-benzenesulfonylbenzotriazole were tested on epimastigote and trypomastigote forms demonstrating that derivative had an *in vitro* growth inhibitory dose-dependent activity against epimastigotes. Particularly, after incubation for 72 h, the parasite number in epimastigotes form decreases 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 any anti-trypansomal activity. Compound (**20**)

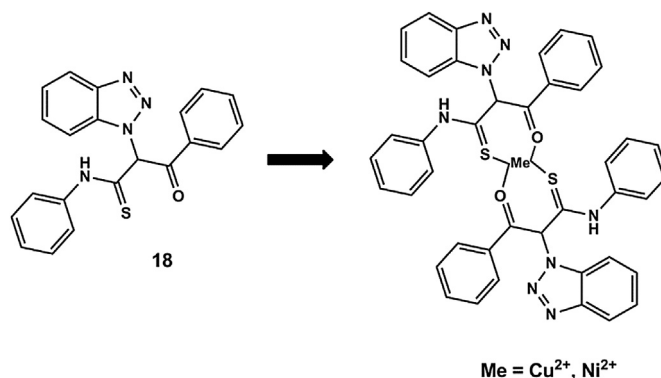


Fig. 15. Solid benzotriazole complexes endowed with antimicrobial activity.

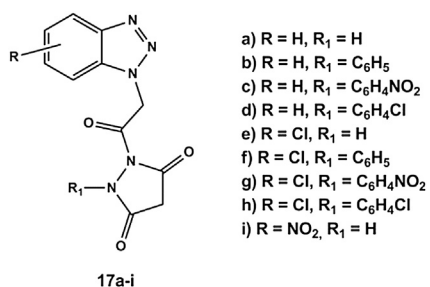


Fig. 14. 1-(2-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)acetyl)-2-*R*<sub>1</sub>-pyrazolidine-3,5-dione derivatives.

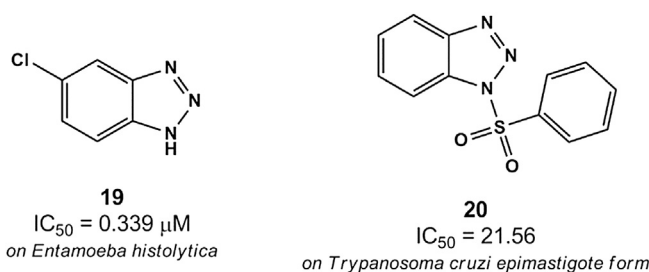


Fig. 16. Benzotriazole derivatives as *Entamoeba histolytica* (**19**) and *Trypanosoma cruzi* (**20**) inhibitors.

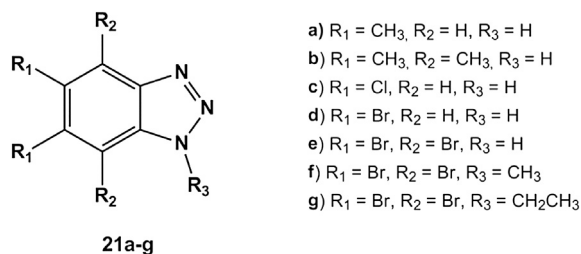


Fig. 17. Substituted benzotriazoles and their N-alkyl derivatives as *Acanthamoeba castellanii* inhibitors.

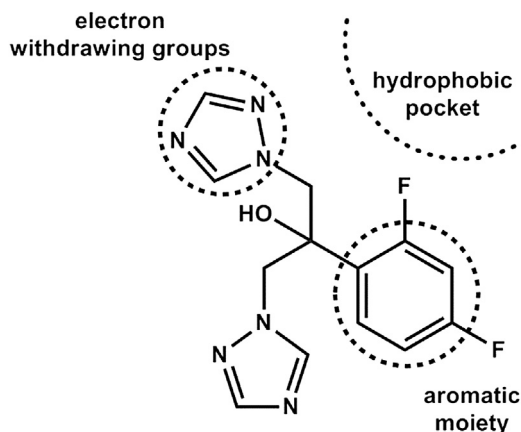


Fig. 18. Schematic view of the key interaction between fluconazole and its receptor.

appeared to be even more effective on trypomastigotes, the infective form of the parasite. Indeed, in earlier time points, a concentration of 50  $\mu\text{g}/\text{mL}$  of this compound induced a trypomastigotes dead 95% higher than in epimastigote forms. On the contrary, BT was less effective, causing only 21% of dead parasites at the same concentration [62].

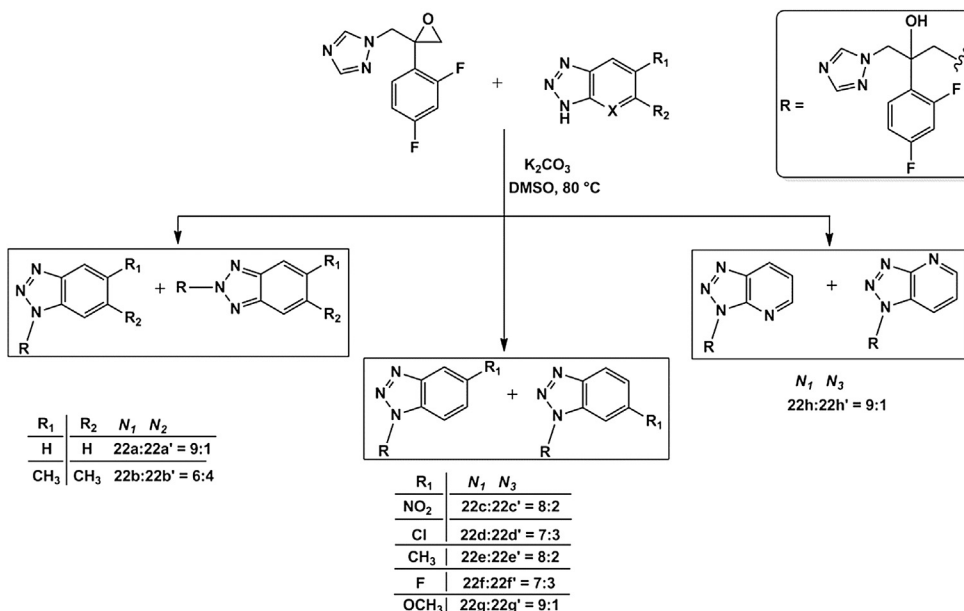


Fig. 19. Synthesis scheme of 5,6 substituted benzotriazoles derivatives endowed with antifungal activity.

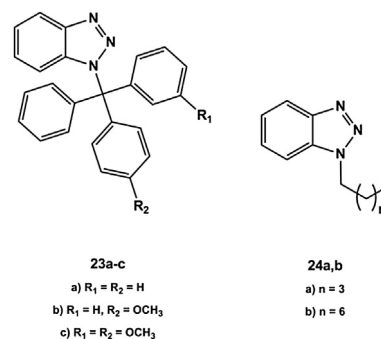


Fig. 20. Chemical structure of the 1,2,3-benzotriazole derivatives proposed by Rezaei et al.

On the basis of the structure of 4,5,6,7-tetrabromo-1*H*-benzotriazole (TBBt), a highly selective inhibitor of protein kinase 2 (CK2) [63], bromo-, chloro- and methyl-analogues of benzotriazole and their N-alkyl derivatives (**21a–g**) (Fig. 17) were synthesized and evaluated for their *in vitro* antiprotozoal activity against *Acanthamoeba castellanii* trophozoites and cysts [64]. This protozoa, as well as several species of free-living amoebae belonging to the genus *Acanthamoeba*, represents a serious risk to human health [65]. Many of the synthesized compounds showed antiprotozoal activity comparable to the currently used drug chlorhexidine. Derivatives (**21a**) and (**21d**) showed even higher activity, especially towards *Acanthamoeba* cysts.

### 3. Benzotriazole as antimycotic agent

As previously reported, several authors disclosed the biological evaluation of imidazole derivatives and benzotriazole analogues as antibacterial and antimycotic agents. Unfortunately, in most cases a selectivity of action was not demonstrated [51,54]. However, the structural model of the best-known antifungal drug fluconazole offers an interesting starting point for drug design studies. Indeed, the triazolic system can be replaced by a benzotriazole ring, in order to evaluate the effects of the bioisosteric replacement on the

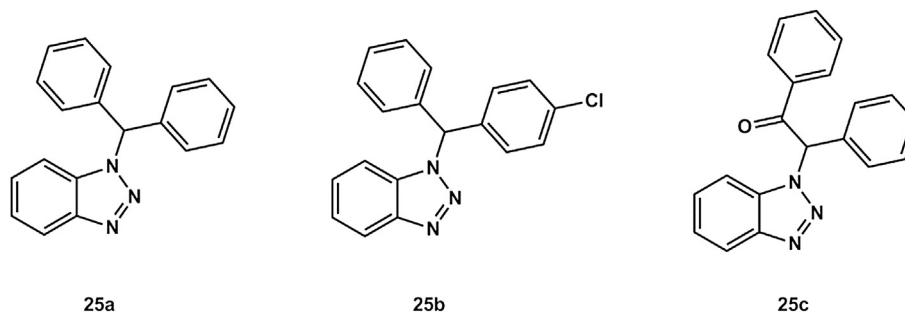


Fig. 21. Biphenyl and benzhydryl benzotriazole derivatives.

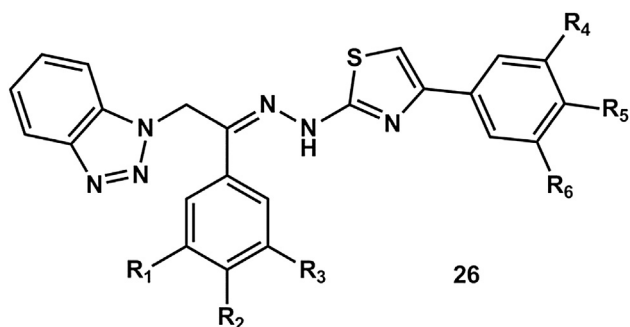


Fig. 22. Benzotriazol-1-yl-1-(phenyl)ethylidene)hydrazinyl)-4-phenylthiazole derivatives chemical structure.

biological behavior. Concerning the mechanism of action, it is known that antimycotic drugs, such as fluconazole [66], itraconazole [67], voriconazole [68], ravuconazole [69] and posaconazole [70] exert their pharmacological action by inhibiting the fungal 14  $\alpha$ -demethylase cytochrome P450. Known as CYP51, this is an essential enzyme in the biosynthesis of sterols. In particular CYP51 removes the 14  $\alpha$ -methyl group of lanosterol using oxygen and NADPH by oxidation, transforming it into ergosterol, an essential component of the fungal cell membranes [71]. These drugs act by displacing lanosterol from CYP51 binding site, causing a block in the biosynthesis of ergosterol and an accumulation of 14  $\alpha$ -methylsterols [72].

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, which 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 [73], as described in Fig. 18.

Fluconazole is effective against candidiasis after both oral and parenteral administration, but it is ineffective against aspergillosis. Besides, it was observed an increasing incidence of infections caused by *Candida not albicans* (such as *Candida glabrata* and *Candida krusei*) resistant to this drug [74]. These evidences have greatly stimulated the pharmaceutical research in the antimycotic field, leading to the synthesis of new derivatives having a benzotriazole group as a central structure. These compounds, in comparison with imidazole antifungal drugs, seem to possess a broader antifungal spectrum paired with a lower toxicity [75]. From this perspective, Patel's research group elided a 1,2,4-triazole ring of fluconazole and replaced it with a 5(6)-(un)substituted benzotriazole, leading to compounds (22a–h) and (22a'–h') (Fig. 19) [76].

Particularly, compounds (22b'), (22d) and (22e') exhibited potent antifungal activity, with MICs values on *Candida albicans* ranging from 1.6  $\mu\text{g/ml}$  to 25  $\mu\text{g/ml}$ . Moreover, the introduction on the benzotriazole ring of the small hydrophobic groups –Cl, –CH<sub>3</sub> and di–CH<sub>3</sub> led to compounds successful against both *Candida* and *Aspergillus* spp., with 12.5–25  $\mu\text{g/ml}$  MIC values on *Aspergillus niger*. Replacement of the benzotriazole ring with a triazolopyridine generate polar and less potent analogues (22h) and (22h'). Finally, introduction of electron-withdrawing groups such as –NO<sub>2</sub>, –Cl, –F, in position 5 of the benzotriazole moiety increased the antimycotic activity. On the contrary, the same substitution in position 6 led to compounds definitely less potent.

The 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) was reported. Indeed, they showed an antimycotic activity higher

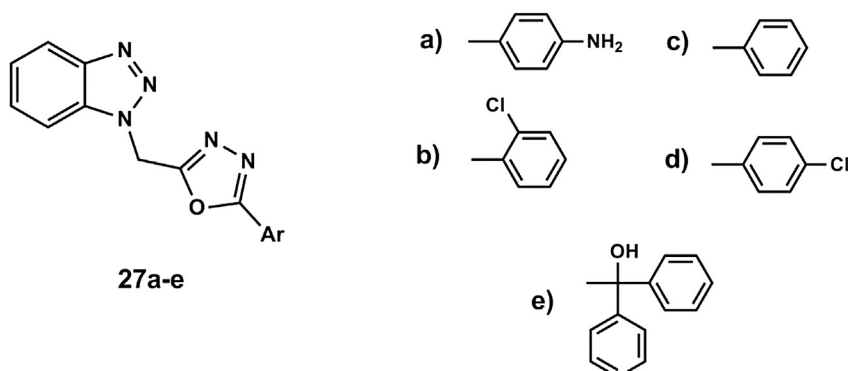


Fig. 23. Benzotriazol-1-yl-methyl-5-substituted-1,3,4-oxadiazole derivatives synthesized by Saini et al.



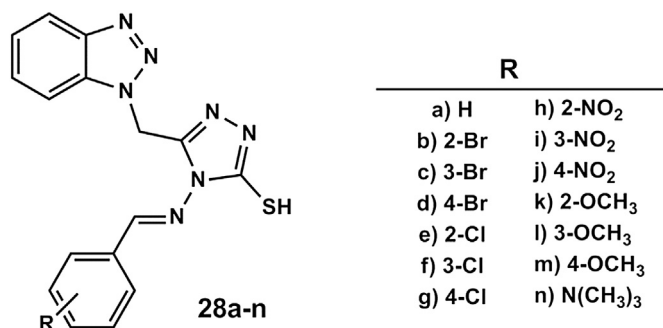


Fig. 24. Schiff bases of benzotriazolyl-4-amino-1,2,4-triazoles.

than the respective R enantiomers. Furthermore, to evaluate interaction differences at the binding site, CYP51 amino acid sequences of *Mycobacterium Tuberculosis* and *Aspergillus niger* have been compared. Obtained results showed antifungal potency differences among benzotriazole regioisomer couples. These differences were mainly due to the triazole heterocycles placement into MT-CYP51 active site.

Also Rezaei and colleagues synthesized an assortment of triazole derivatives, including benzotriazole, as CYP51 inhibitors. The chemical structure of benzotriazole derivatives (**23a–c**) and (**24a,b**) is displayed in Fig. 20. For all synthesized compounds, a molecular docking simulation on CYP51 active site was performed.

Collected data resulted comparable to those obtained using fluconazole. In particular, lower final docking energy (FDE) values, index of better drug–target interaction, were obtained for 1,2,4 triazole derivatives. However, there is no correlation between antifungal activity and FDE. These analyses underlined a relationship between antimicrobial potency and molecular penetration into fungi cell. Consequently, the presence of bulky substituents, as OCH<sub>3</sub>, was believed to be responsible for the activity decrease [77,78].

In following research, the team headed by Rezaei continued the investigation on CYP51 inhibitors synthesizing new 1,2,4-triazole, imidazole, benzimidazole and benzotriazole derivatives (**25a–c**) (Fig. 21). Throughout drug-design studies, applying Autodock on MT-CYP51, the best *in silico* promising derivatives were synthesized and their antimycotic activity was compared to that of fluconazole and itraconazole, used as reference drugs [78]. Unfortunately, benzotriazole derivatives did not show antifungal activity against *Candida* species. Exceptionally, these compounds were endowed with activity against the dermatophyte *Microsporum canis*. Only some imidazole or 1,2,4-triazole derivatives appeared active compounds against all microbial species taken into account. This behavior was probably due to the presence of the five-membered nitrogen heterocyclic ring and to their lower size, which allowed best access to the fungal cells.

Related to the same azole antifungal model, new compounds (**26**) were produced by Gaikwad and colleagues, wherein the benzotriazole core was modified by alkylation with complicated side chains bearing a substituted thiazole ring, as shown in Fig. 22. Tested on a wide number of microorganisms, Gram-positive, Gram-negative and fungi, in all cases the best activities were observed for derivatives bearing electron withdrawing substituents as F, Cl, Br, and NO<sub>2</sub> on side benzene phenyl groups. To be precise, these derivatives were able to equally inhibit the growth of Gram-positive and Gram-negative bacteria, as well as *Candida albicans* and *Aspergillus niger* growth [79].

Modification on the side chain of BT led Saini's group to develop new substituted 1,3,4-oxadiazole derivatives (**27a–e**), depicted in Fig. 23. All compounds demonstrated to possess good anti-fungal activity, and 2'-chloro substituted derivative (**27b**) was more active than unsubstituted (**27c**) and alkyl substituted (**27e**) analogues [80].

Numerous Schiff bases of benzotriazolyl-4-amino-1,2,4-triazoles (**28a–n**, Fig. 24) were investigated by Prasad et al. All derivatives were screened for antifungal activity using cup plate agar diffusion method against *Candida albicans*, and compound (**28d**) had the most potent antifungal effect [81].

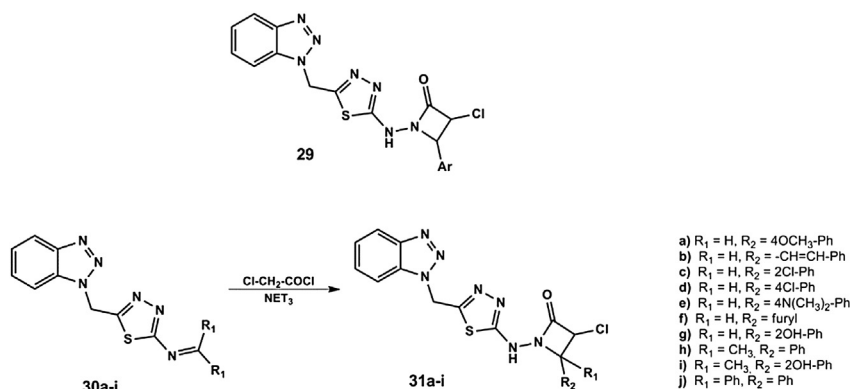


Fig. 25. Benzotriazole bearing antifungal and anticonvulsant activity.

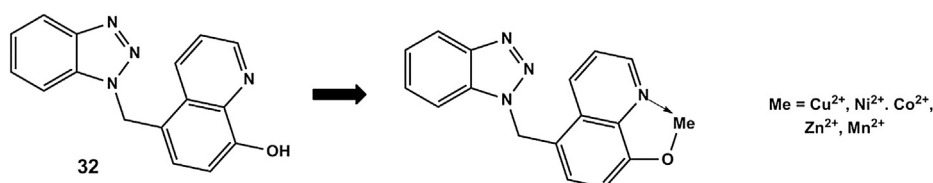


Fig. 26. Metal chelates of 5-[1(H)-Benzotriazole methylene]-8-quinolinol.

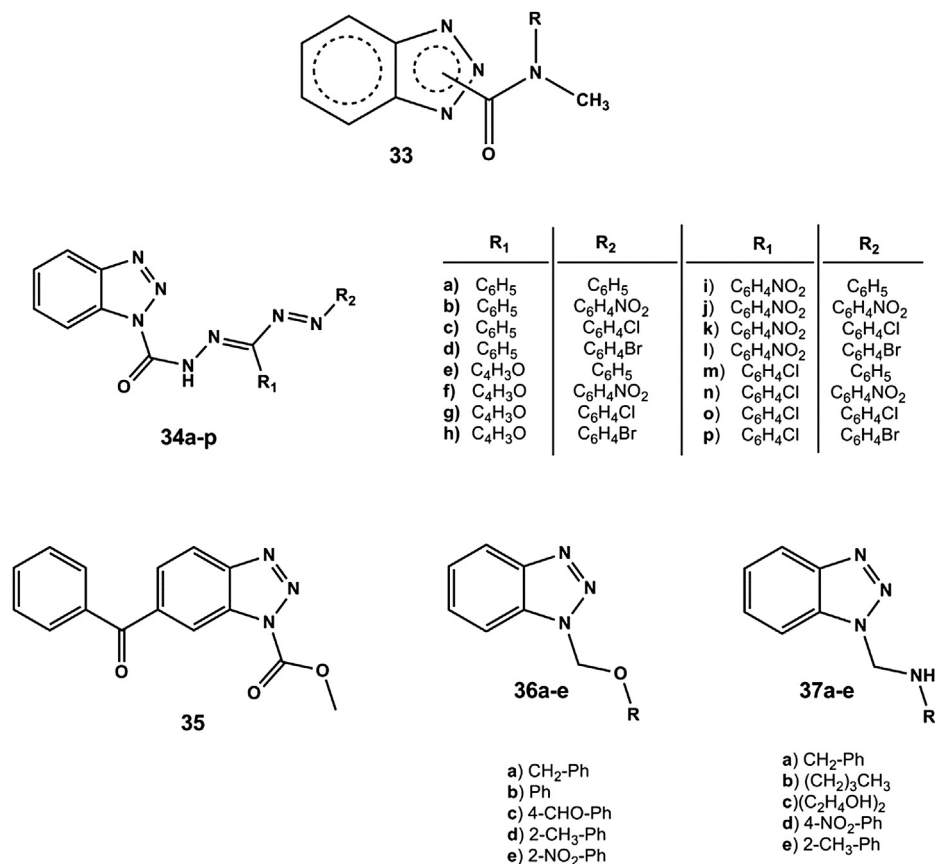


Fig. 27. Benzotriazole derivatives endowed with anthelmintic activity.

Moreover, compounds bearing simultaneously benzotriazole, thiaziazole and azetidone moieties (**29**) were reported by Shukla and Srivastava for their antifungal and antibacterial activities [82]. Similar compounds (**31a–i**) and their precursors (**30a–i**) were more extensively examined by Srivastana and colleagues. All compounds are depicted in Fig. 25. They investigated not only their antimicrobial behavior, but also their anticonvulsant activity. Compound (**31c**) stood out, being able to protect 100% of the

induced seizures, in the same way as reference drug phenobarbitone [83].

Antifungal activity was revealed even for complexes of 5-[(1*H*-benzotriazol-1-yl)methyl] 8-quinolinol (**32**). Different transition metals formed complexes with this compound, as depicted in Fig. 26, and some authors demonstrated that the simple ligand is less toxic for various type of fungi than its chelates. Particularly, Cu<sup>2+</sup> chelate resulted in one of the most effective derivative [84].

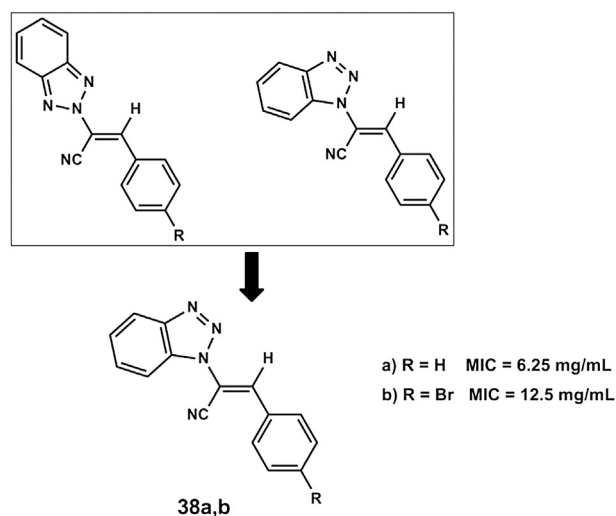
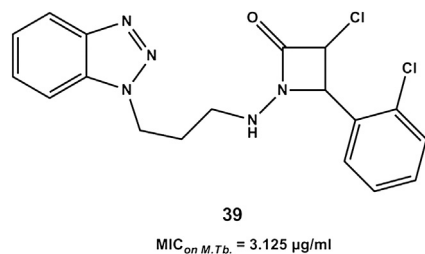


Fig. 28. 3-Aryl substituted-2-[1*H*(2*H*)benzotriazol-1(2)-yl]acrylonitriles general formula and MIC values reported for compounds **38a,b**.

#### 4. Benzotriazole as anthelmintic agent

Worm infections are the most common infections worldwide [85]. They affect the poorest and most deprived communities and are recognized as cause of chronic ill-health amongst the people living in tropical and subtropical areas [86]. Actually, the benzimidazole nucleus is the core of the anthelmintics with the broadest known spectrum of activity [2]. Moreover, a similar activity is reported also for some benzotriazole derivatives, above all benzotriazoles with 1- and 2-carbamoyl substituents (**33**, Fig. 27) [87].

In this connection a series of benzotriazole-1-carbonyl-3,5-arylfornazans (**34a–p**) was synthesized by Sudhir *et al.* under ultrasonic and solvent free conditions. All compounds were tested for activity against adult earthworm *Pheretima posthuma* using mebendazole and albendazole as reference drugs. Although none of the tested compounds were more effective than the reference drugs, derivatives (**34b,f,j,n**) showed dose-dependent anthelmintic activity. This behavior was attributed to the *p*-nitrophenyl substituent attached to azo group of benzotriazole moiety.



**Fig. 29.** 2-Oxo-4-substituted aryl-azetidinone derivative of benzotriazole.

Moreover, an additional *p*-nitrophenyl group, as in (**34j**), provided the best results [88].

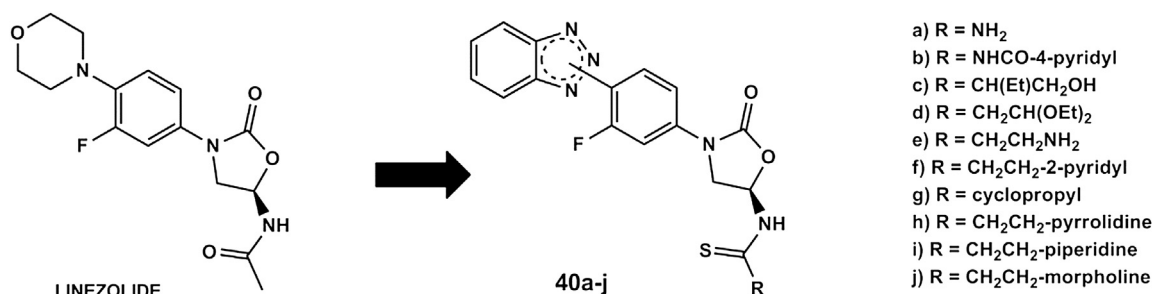
On the contrary, methyl 6-benzoyl-1*H*-benzo[d][1,2,3]triazole-1-carboxylate (**35**) was designed to be active against *Necator americanus* infections but resulted totally inactive in newborn hamsters.

Finally, N1alkyl/aryl (**36a–e**) and alkoxy/aryloxy (**37a–e**) arylaminomethylene benzotriazoles have been described as good anthelmintic agents on *Pheretima posthuma*. The anthelmintic activity of all derivatives was comparable with the reference drug Albendazole. Moreover, alkoxy/aryloxymethylene (**37a–e**) benzotriazoles resulted more potent than the alkyl/aryl aminomethylene analogues (**35a–e**) [89].

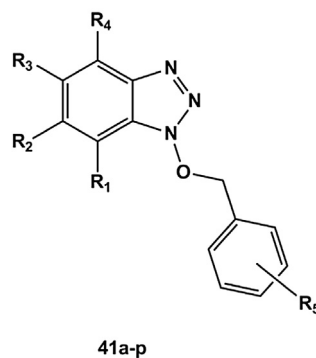
## 5. Benzotriazole as antimycobacterial agent

Despite the perception, nowadays Tuberculosis (TB) is still one of the most common infectious diseases, estimating that as much as one-third of world's population (~2 billion people) is infected with *Mycobacterium tuberculosis* (*M. tb*) [90]. Annually about nine million new TB cases occur and up to two million people die from this disease [91]. Even more frightening is the rapid emerge and spread of Multi-Drug-Resistant (MDR) [92] and Extensively-Drug-Resistant (XDR) TB strains [93] in all regions of the world, along with the deadly synergy of Human Immunodeficiency Virus (HIV) and *M. tb* [94,95]. Worldwide, TB is the leading cause of death among HIV- positive people [96].

The treatment of active tuberculosis require a combination of several drugs, such as isoniazid, pyrazinamide, ethambutol, rifampicin and streptomycin [97]. All of them are used in different combinations as first-line therapy [98]. On the contrary, quinolones are classified as second-line drugs [99], even if their use is recommended for the management of MDR-TB [100]. However, due to the outcoming of MDR and XDR-TB strains, worldwide there is an urgent need for new drugs acting through novel mechanisms of action from those mentioned above. Moreover, new drugs could be useful to develop regimens shorter than the standard 20-month treatment regimen.

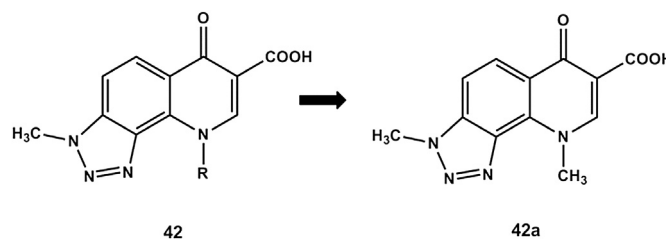


**Fig. 30.** Bisosteric substitution on the structure of linezolid.



- a) R<sub>1</sub> = R<sub>4</sub> = H, R<sub>2</sub> = R<sub>3</sub> = Br, R<sub>5</sub> = 3-NO<sub>2</sub>
- b) R<sub>1</sub> = R<sub>4</sub> = H, R<sub>2</sub> = R<sub>3</sub> = Br, R<sub>5</sub> = 3,5-NO<sub>2</sub>
- c) R<sub>1</sub> = R<sub>4</sub> = H, R<sub>2</sub> = R<sub>3</sub> = Br, R<sub>5</sub> = 2,4-NO<sub>2</sub>
- d) R<sub>1</sub> = R<sub>2</sub> = R<sub>4</sub> = H, R<sub>3</sub> = Br, R<sub>5</sub> = 3-NO<sub>2</sub>
- e) R<sub>1</sub> = R<sub>2</sub> = R<sub>4</sub> = H, R<sub>3</sub> = Br, R<sub>5</sub> = 3,5-NO<sub>2</sub>
- f) R<sub>1</sub> = R<sub>4</sub> = H, R<sub>2</sub> = R<sub>3</sub> = Cl, R<sub>5</sub> = 2-NO<sub>2</sub>
- g) R<sub>1</sub> = R<sub>4</sub> = H, R<sub>2</sub> = R<sub>3</sub> = Cl, R<sub>5</sub> = 3-NO<sub>2</sub>
- h) R<sub>1</sub> = R<sub>4</sub> = H, R<sub>2</sub> = R<sub>3</sub> = Cl, R<sub>5</sub> = 4-NO<sub>2</sub>
- i) R<sub>1</sub> = R<sub>4</sub> = H, R<sub>2</sub> = R<sub>3</sub> = Cl, R<sub>5</sub> = 3,5-NO<sub>2</sub>
- j) R<sub>1</sub> = R<sub>4</sub> = H, R<sub>2</sub> = R<sub>3</sub> = Cl, R<sub>5</sub> = 2,4-NO<sub>2</sub>
- k) R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = Br, R<sub>5</sub> = 3,5-NO<sub>2</sub>
- l) R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = Cl, R<sub>5</sub> = 2-NO<sub>2</sub>
- m) R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = Cl, R<sub>5</sub> = 3-NO<sub>2</sub>
- n) R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = Cl, R<sub>5</sub> = 4-NO<sub>2</sub>
- o) R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = Cl, R<sub>5</sub> = 3,5-NO<sub>2</sub>
- p) R<sub>1</sub> = R<sub>2</sub> = R<sub>3</sub> = R<sub>4</sub> = Cl, R<sub>5</sub> = 2,4-NO<sub>2</sub>

**Fig. 31.** O-nitrobenzylated derivatives of halogenosubstituted 1-hydroxybenzotriazoles.



**Fig. 32.** Chemical structure of 3,9-dimethyl-6-oxo-6,9-dihydro-3*H*-[1,2,3]triazolo[4,5-*h*]quinoline-7-carboxylic acid.

Several groups focused their attention and research efforts on this field and a large amount of molecules have been tested for this purpose [101–104]. Since '90s Carta and co-workers focused their attention on the bioisosteric modification of quinolones. As result, various benzotriazole derivatives were synthesized. A first series of 3-aryl substituted-2-(1*H*(2*H*)benzotriazol-1(2-yl)acrylonitriles [105] was prepared with the aim to identify the best substituents on the aryl moiety. Groups holding various electron-acceptor or donor properties and lipophilic–hydrophilic balance were opportunely selected. During the synthesis, the performed Knoevenagel condensation in most cases resulted in the formation of only *E*-isomers, and only in a few cases in 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 (**38a**) outcome the best option, exhibiting the highest antimycobacterial activity *in vitro*, also against *Mycobacterium avium*. The only exception is represented by 4-bromophenyl derivative (**38b**), although its activity resulted lower than the one of (**38a**), as depicted in Fig. 28.

With the aim to improve the antimycobacterial activity, further modifications were performed, such as the introduction in the

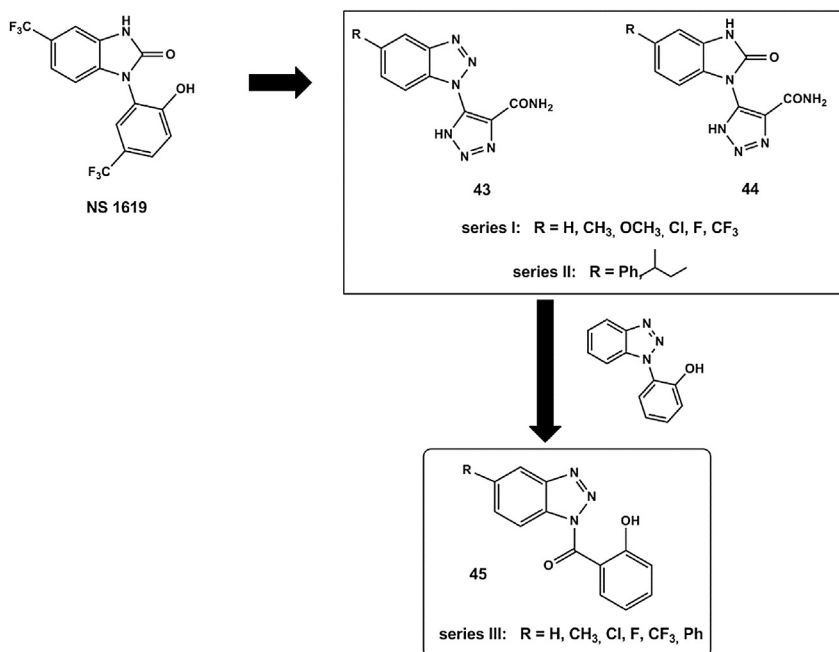


Fig. 33. Potential activators of potassium channel opener.

phenyl ring C-4 of two or more electron-releasing groups, or the replacement of the phenyl ring with cyclohexyl or larger aromatic rings [106]. Unfortunately the produced 3-aryl-, 3-cyclohexyl and 3-heteroaryl substituted-2-(1*H*(2*H*)-benzotriazol-1(2)-yl)prop-2-enitriles, prop-2-enamides and propenoic acids showed a strong reduction or a loss of the activity in spite of their increased lipophilic character. This indicates that the steric hindrance, as well as the nature of the substituents, 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 alternatively introduced at position 2', 3' and 4' in the phenyl moiety [107]. Both the previously synthesized and the new derivatives cytotoxicity was evaluated against MT-4 cells. Unluckily, the relevant cytotoxicity showed by many derivatives induced the group to dispose of acrylonitriles as antitubercular and to evaluate them as antiproliferative agents, as reported above.

Nevertheless, benzotriazole remains an interesting heterocycle for its antimycobacterial proprieties, and various groups tried to combine its chemical structures within well known antibacterial agents. Therefore, with the aim to develop new antitubercular

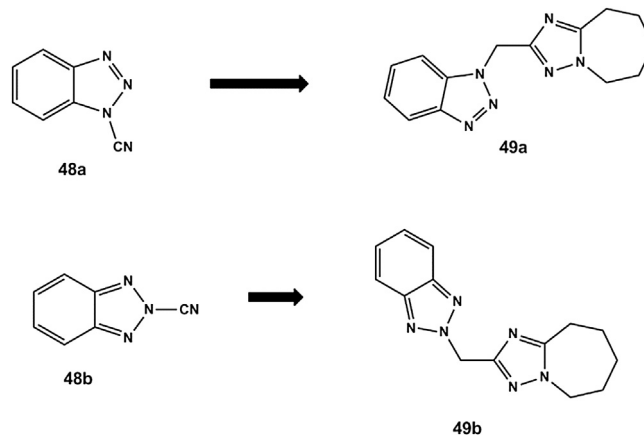


Fig. 35. Benzotriazol-1(2)-yl-tetrahydro-triazoloazepine.

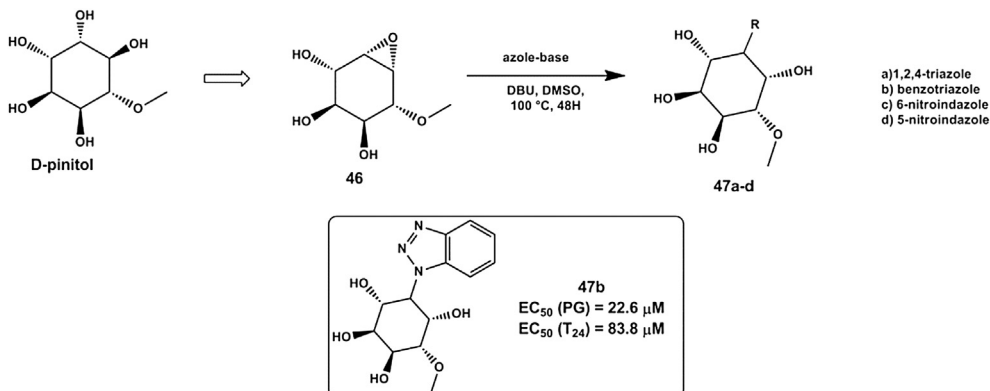


Fig. 34. 3'-O-Methyl-5'-deoxy-5'-(1,2,4-triazole-1-yl)-D-chiro-inositol.

agents able to succeed toward *M. tb* sensitive and resistant strains new molecular series were designed and synthesized.

Dubey et al. coupled the benzotriazole proprieties with the antimicrobial activities of the  $\beta$ -lactams 2-azetidiones [108,109]. The 2-oxo-4-substituted aryl-azetidione derivatives of benzotriazole were prepared by both conventional and microwave irradiation, the latter outcoming the best performing synthetic way. All prepared compounds showed no cytotoxicity and were tested against *M. tb* and some other microorganisms, such as bacteria and fungi. As result, compound (39), emerged, showing a promising activity against *M. tb* (MIC = 3.125  $\mu$ g/ml). Moreover, it resulted active against some bacteria (MIC = 0.1  $\mu$ g/ml on *Escherichia coli*) and fungi (MIC = 0.5  $\mu$ g/ml on *Aspergillus niger*), (Fig. 29). For this class of compounds no selectivity of action is reported.

Dixit et al. used the Linezolid structure as basis scaffold to create a new class of oxazolidinones derivatives [110] (Fig. 30), performing a bioisosteric substitution of the morpholinic moiety with the benzotriazole structure. Ten 1-[3-(4-benzotriazol-1/2-yl-3-fluorophenyl)-2-oxo-oxazolidin-5-ylmethyl]-3-substituted-thiourea derivatives (40a–j) 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). Compound (40g) stood out, showing an excellent *in vitro* activity against *M. Tb*. H37Rv (MIC = 0.06  $\mu$ M) and both drug sensitive and resistant clinical isolates (MIC = 0.125–1.0  $\mu$ M and 1–2  $\mu$ M, respectively). These MIC values were equivalent to linezolid and superior to isoniazid against all strains.

Ewa's group presented a series of benzotriazoles derivatives. Their work was based on the considerable antimycobacterial activity revealed for benzimidazole derivatives modified both in the heterocyclic core and in exocyclic constituents. Particularly, it was observed that the biological activity was enhanced by the introduction of a nitrobenzylsulfenyl group at position 2 and by a substitution on the heterocycles benzene moiety with halogen atoms [111–114].

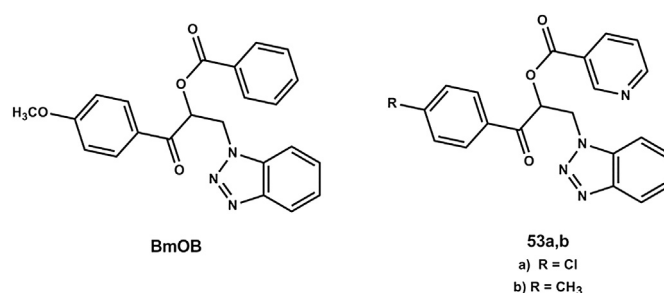


Fig. 37. Chemical structure of BmOB and nicotinic derivatives 51a,b.

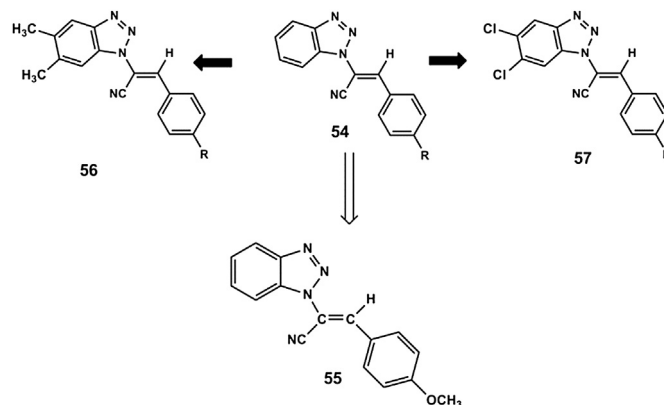


Fig. 38. Benzotriazol acrylonitriles: structure and lead compound of the series, (*E*)-2-(1*H*-benzo [d][1,2,3]triazol-1-yl)-3-(4-methoxyphenyl)acrylonitrile.

Several new O-nitrobenzylated derivatives of halogeno-substituted 1-hydroxybenzotriazoles (41a–p) (Fig. 31) were then synthesized and their activity was tested against four Mycobacterium strains (a standard strain of *M. tb* H37Rv, an INH-resistant *M. tb* strain - clinical isolate -, *Mycobacterium avium* intercellular

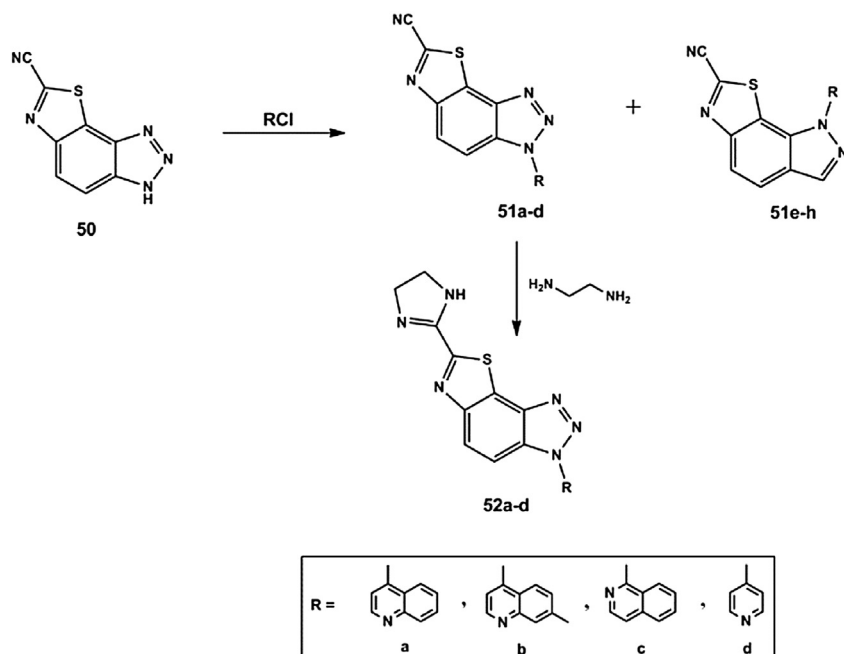


Fig. 36. Cyano thiazole-benzotriazoles derivatives.

complex and IHN-resistant MOTT *Mycobacterium kansasii* strain) [115]. 5,6-dichloro-1-(3,5-dinitrobenzyloxy)-1*H*-benzotriazole (**41i**) emerged for its activity against the reference strain H37Rv, with MIC values comparable with those of reference drug isoniazid. Even *Mycobacterium kansasii* and *M. tb* 1753 (isoniazide-resistant) were moderately sensitive to this 5,6-dichloro derivative.

As observed until now, the applied strategies to design a new antitubercular agent normally scheduled the bioisosteric modification of a known 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 [37,116,117], 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 infections and, to a lesser extent, in case of severe adverse reactions to the conventional antituberculous regimen [118]. However, they are still classified as second-line drugs, since their use in tuberculosis treatment remains controversial [99]. This behavior is justified by the fact that the emergence of fluoroquinolone resistance in MDR strains is possible [119], 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 [120].

Quinolones mechanism of action and pharmacokinetics are widely studied, and various review can be found in literature [121–124]. 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 against *M. tb* sensitive and resistant strains [37,116,117]. As reported by Milata review [125], the Carta's group discovered that only few triazolo[4,5-*h*]quinolone carboxylic acids exhibited interesting low MIC<sub>90</sub> values (5.0–1.6 µg/mL), observing also that the activity was related to the length and position of the substituent at triazole-nitrogen. Compounds bearing methyl group at N3 showed the higher activity. This first observation prompted the group to focalize their work. They designed and selectively synthesized a series of 3-methyl-9-substituted-6-oxo-6,9-dihydro-3*H*-[1,2,3]-triazolo[4,5-*h*]quinolone-carboxylic acids (**42**) (Fig. 32). A variety of substituents on the quinolone nitrogen were introduced with the aim to improve the biological activity. All derivatives were tested against *M. tb* H37Rv and further 11 clinically isolated strains endowed with a wide spectrum of drug resistance. Derivative (**42a**) stood out as the most potent derivative, exhibiting MIC<sub>90</sub> = 0.5 µg/mL against all tested strains.

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 0.5 and 0.25 µg/mL of lead compound. Both macrophage cultures were lysed 7 days after and

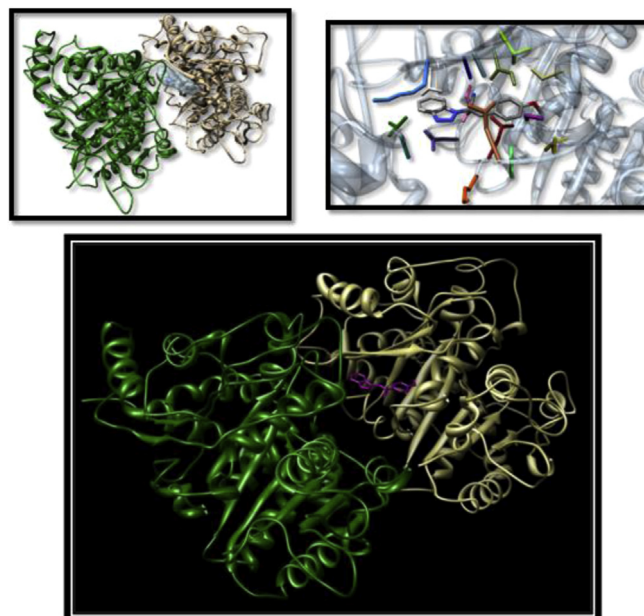


Fig. 40. Interaction of the derivative (*E*)-2-(1*H*-benzo[*d*][1,2,3]triazol-1-yl)-3-(4-methoxyphenyl)acrylonitrile in the binding site of tubulin.

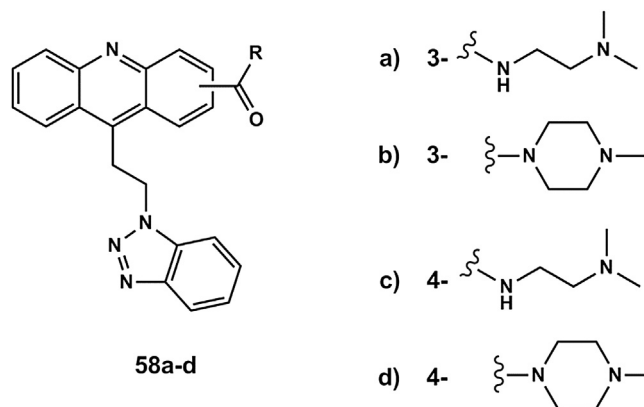


Fig. 41. 9-Aminoacridine derivatives provided by click chemistry.

the mycobacterial growth was estimated about 5000 and 8000 CFU/mL, respectively, while the untreated culture grew regularly. In addition, supplementary analysis revealed good antimycobacterial activity against several *Mycobacterium avium paratuberculosis*, *Mycobacterium smegmatis* and *Mycobacterium bovis* strains [126]. Further modifications of the substituents in N-3 and

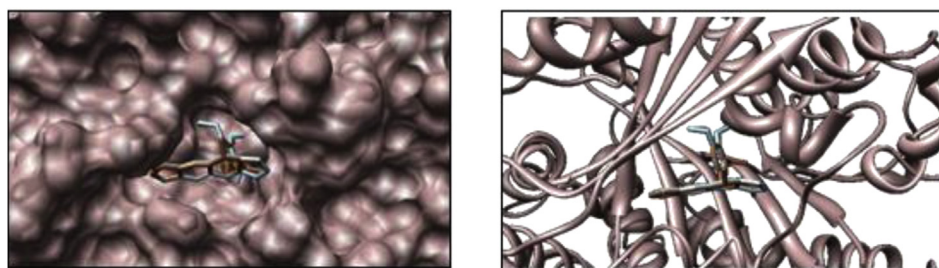
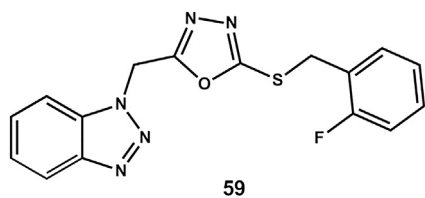
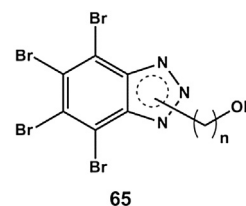


Fig. 39. Validation of the three-dimensional model. Comparison of co-crystallized podophyllotoxin (brown) and subjected to docking (blue) in the allosteric binding site on human  $\beta$ -tubulin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



59



65

Fig. 42. 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-((2-fluorobenzyl)thio)-1,3,4-oxadiazole (59) chemical structure.

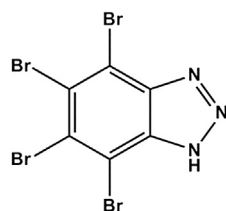
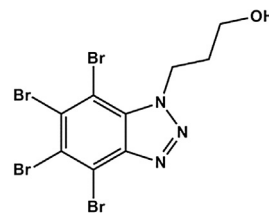
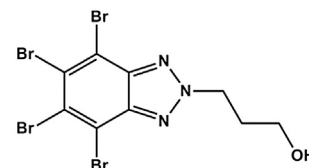
60  
TBBt61  
TBBi

Fig. 43. 4,5,6,7-tetrabromobenzimidazole (TBBi) and 4,5,6,7-tetrabromobenzotriazole (TBBt) chemical structure.



65a

$IC_{50} = 0.48 \mu\text{M}$  [on CK2(a<sub>2</sub>b<sub>2</sub>)]  
 $IC_{50} = 0.32 \mu\text{M}$  [on CK2a]



65b

$IC_{50} = 0.82 \text{ mM}$  [CK2(a<sub>2</sub>b<sub>2</sub>)]  
 $IC_{50} = 0.34 \text{ mM}$  [CK2a]

Fig. 46. TBBt N-hydroxyalkyl derivatives.

N-9 positions of the ring system with bulking groups do not improved the biological activity. Comparing new derivatives with previous series, methyl group was confirmed to be the most effective substituent in both positions [127]. Lastly, when MICs were determined against a panel of Gram-positive and Gram-

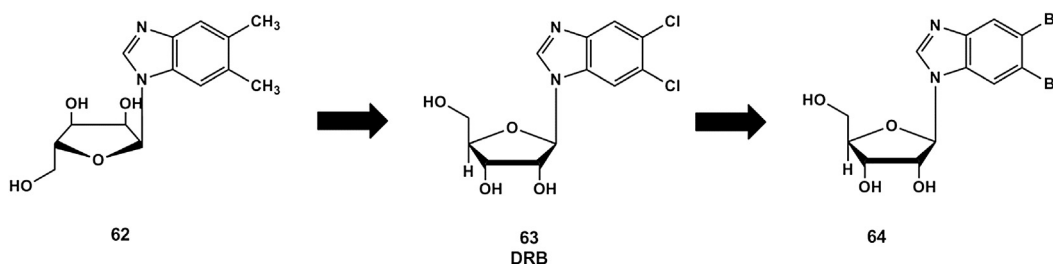


Fig. 44. 1- $\alpha$ -Ribofuranosido-5,6-dimethylbenzimidazole derivatives.

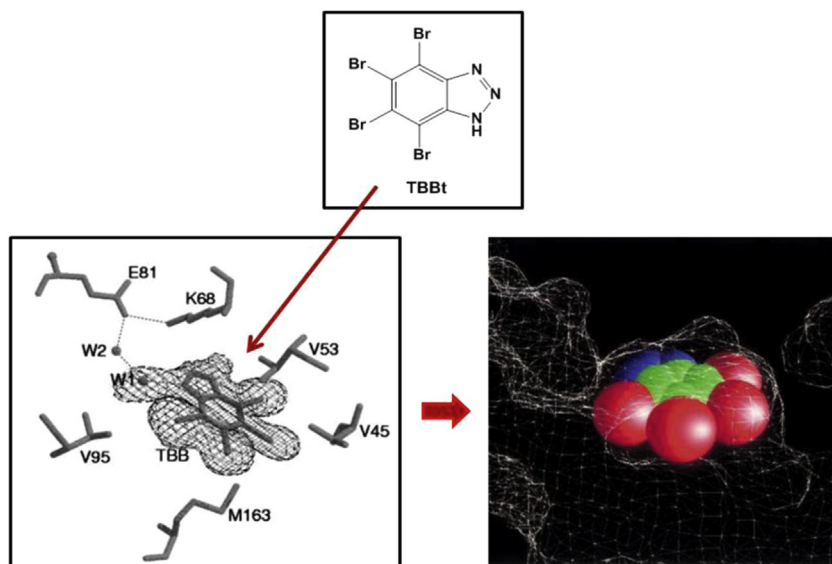


Fig. 45. TBBt inside CK2 $\alpha$  hydrophobic binding pocket.

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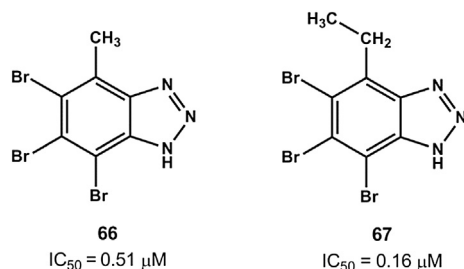


Fig. 47. 5,6,7-tribromo-4-methyl-1H-benzotriazole and 5,6,7-tribromo-4-ethyl-1H-benzotriazole chemical structures.

negative bacteria as well as against *Candida* sp., all compounds resulted inactive (MIC = 64–100 μg/mL). For this new class of quinolones these results suggested a specific antimycobacterial potential. This behavior was probably due to a different mechanism of action toward quinolones, leading them to be good candidates for further developments [128].

## 6. Benzotriazole as potassium channel activator

Potassium channels activators represent an emerging class of drugs for the treatment of nervous, respiratory and cardiovascular

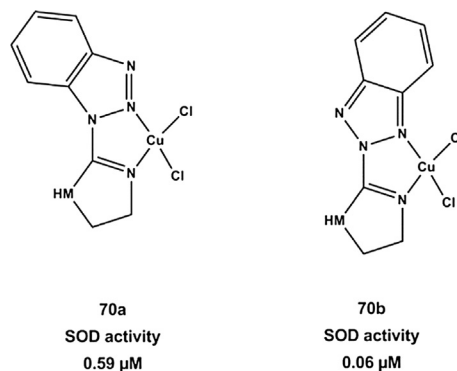


Fig. 50. Chelating bidentate benzotriazole Copper (II) complexes.

disorders; for this reason they represent an interesting research topic in the medicinal chemistry field [129]. From literature, the 1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimidazolone, or NS1619, pop up as a calcium-dependent potassium channels activator. This molecule is able to induce *in vitro* not only cellular hyperpolarization through direct activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channels (BK<sub>Ca</sub> channels), but also direct

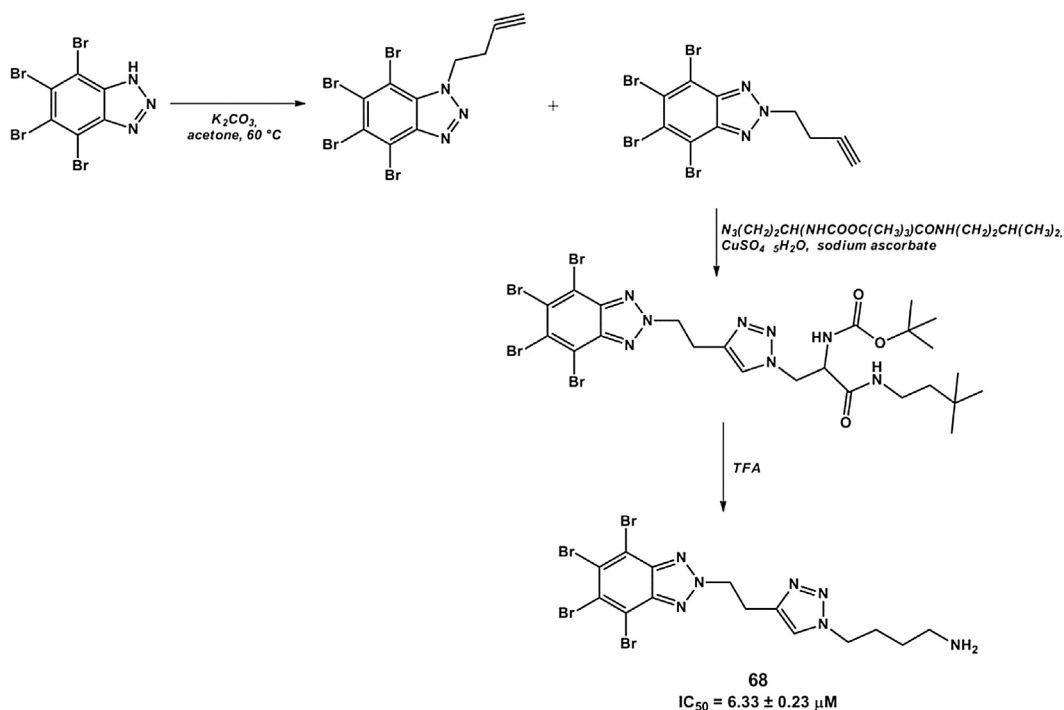


Fig. 48. 4-(4-(2-(Perbromo-2H-benzo[d][1,2,3]triazol-2-yl)ethyl)-1H-1,2,3-triazol-1-yl)butan-1-amine (68) synthesis.

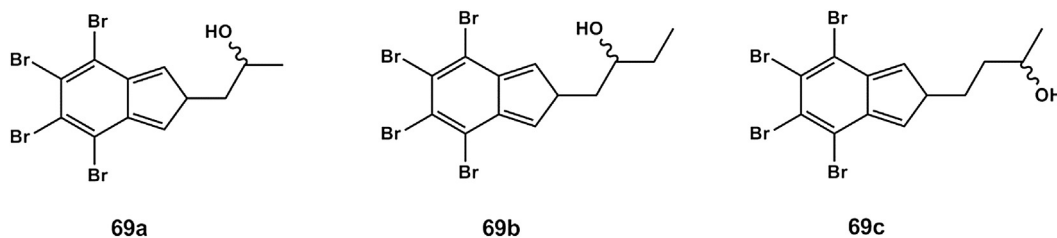


Fig. 49. Optically active hydroxyalkyl derivatives of 4,5,6,7-tetrabromo-1H-benzotriazole.



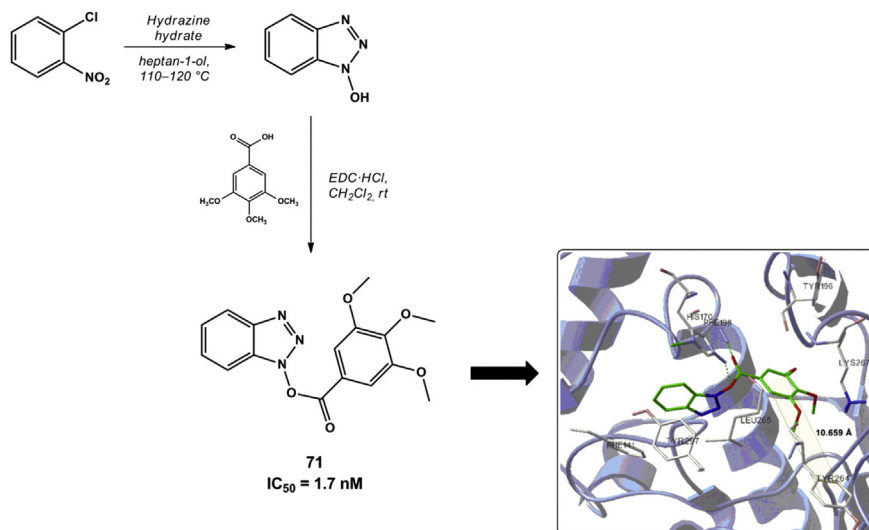


Fig. 51. 1H-benzo[d][1,2,3]triazol-1-yl 3,4,5-trimethoxybenzoate synthesis and binding mode.

inhibition of the  $Ca^{2+}$  channel: both these effects contribute to its vasorelaxant properties [130].

Based on NS1619 properties, the Baragatti and Biagi research group conducted broad studies that primarily led to the design and synthesis of new derivatives series. The 5-substituted-carboxamido-triazolyl-benzotriazoles (**43**), and the corresponding series of 5-substituted-carboxamido-triazolyl-benzimidazolones (**44**) (Fig. 33, series I) were proposed as bioisosteric analogues of NS1619.

All benzotriazole derivatives proved to possess vasorelaxing properties and potency parameters lower than 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 ineffective or inactive. Particularly, SAR studies demonstrated that the absence of substituents on benzotriazole position 5 caused a decrease in potency but not in efficacy. On the contrary, only a methoxy group adversely affected both properties. Furthermore, best results were reported for compounds bearing a simple methyl group. Experimental data suggested a potential relationship between the potency and steric hindrance in this position. Finally, the benzotriazole derivatives lacking the carboxamido group in position 4 of the triazole ring were totally powerless as vasorelaxing agents, demonstrating that the  $CONH_2$  group was an essential structural requirement to exert the vasorelaxing effect due to the potassium channels BK activation [131].

Consequently, analysis was carried out studying the effects of steric hindrance and lipophilicity due to the substituent at position 5. The introduction of bulky and lipophilic groups, such as sec-butyl and phenyl, led to derivatives retaining equivalent potency, even if less potent than derivatives bearing a methyl group in the same position (Fig. 33, **43** 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 derivatives 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 of drugs known as activators of potassium channels [132]. Finally, another series of 5-substituted-1-(2-hydroxybenzoyl)-benzotriazoles (**45**), wherein the triazole group is replaced by a hydroxybenzoyl group, (Fig. 33, series III) was prepared. Thereby, thanks to the bridge determined

by the carbonyl function, new molecules were ensured by greater degree of freedom and therefore flexibility between the two main parts. Biological analysis showed high vasorelaxing potency and efficacy for all derivatives. However, once again, the most potent derivative in this set was the one bearing a methyl group at 5-position, which also showed cardioprotective properties. In contrast, both the introduction of the 2-hydroxyphenyl group in benzotriazole ring position 1 and the replacement of the carbonyl bridge (an electron withdrawing group) with a methylene bridge caused a considerable decrease of the biological activity [133].

## 7. Benzotriazole as antitumor agent

Cancer is actually the second leading cause of death worldwide after cardiovascular diseases, accounting for about 8 million deaths (around 13% of all deaths) [134,135]. The origin of a tumor lies in a somatic cell that has undergone a series of genetic modifications, thus ceasing to respond to normal regulatory mechanisms which operate in a healthy organism. Thereafter, these cells proliferate and form a clone of neoplastic cells, characterized by lack of control proliferation. Concomitantly cells lose potential replicative control and becomes immortal. Additionally, the mechanism for density-dependent inhibition, which usually allows the cells to multiply until they reach a finite cell density (point at which they become quiescent), is deactivated. Tumors are classified in benign and malignant, depending on the biological and morphological characteristics which 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) [136].

The therapeutic approach for the treatment of cancer diseases are different and includes surgical treatment, radiation therapy, immunotherapy or chemotherapy. Polychemotherapy therefore provides the use of virtually toxic drugs, which selectively operate in respect of cancerous cells, thus saving the host healthy cells [137]. Unfortunately, these cells are very similar to each other and due to the difficulty in discriminating between tumor and healthy cells the drugs selectivity is unattainable. In conclusion, even healthy somatic cells are exposed to toxic effects, especially those continuously in mitosis, such as hair follicles, intestinal epithelium and bone marrow [138].

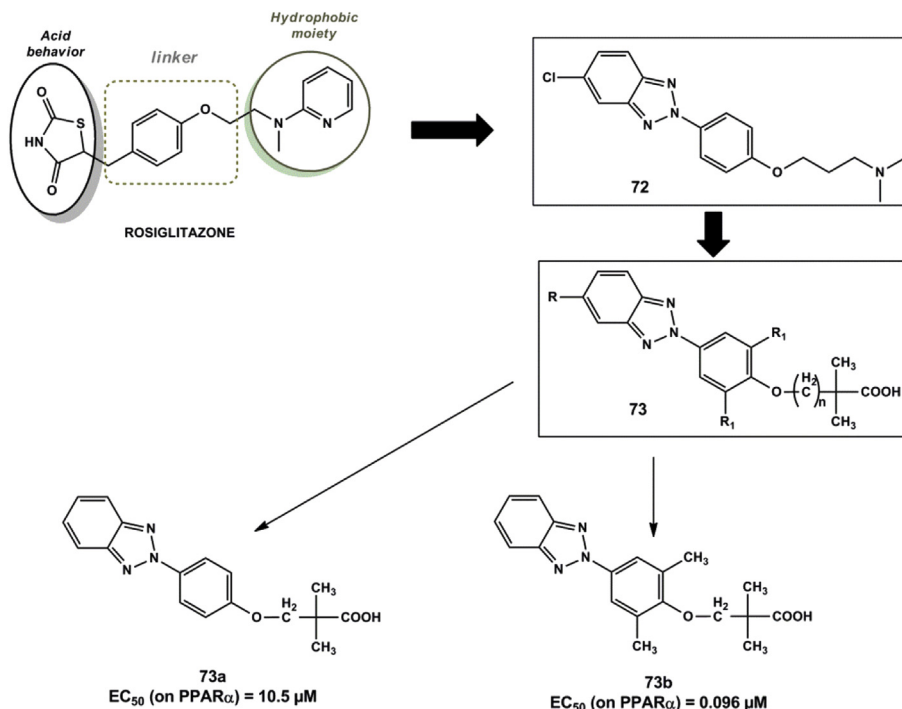


Fig. 52. Benzotriazol-2-yl-phenoxy-2,2-dimethylpropanoic acid derivatives.

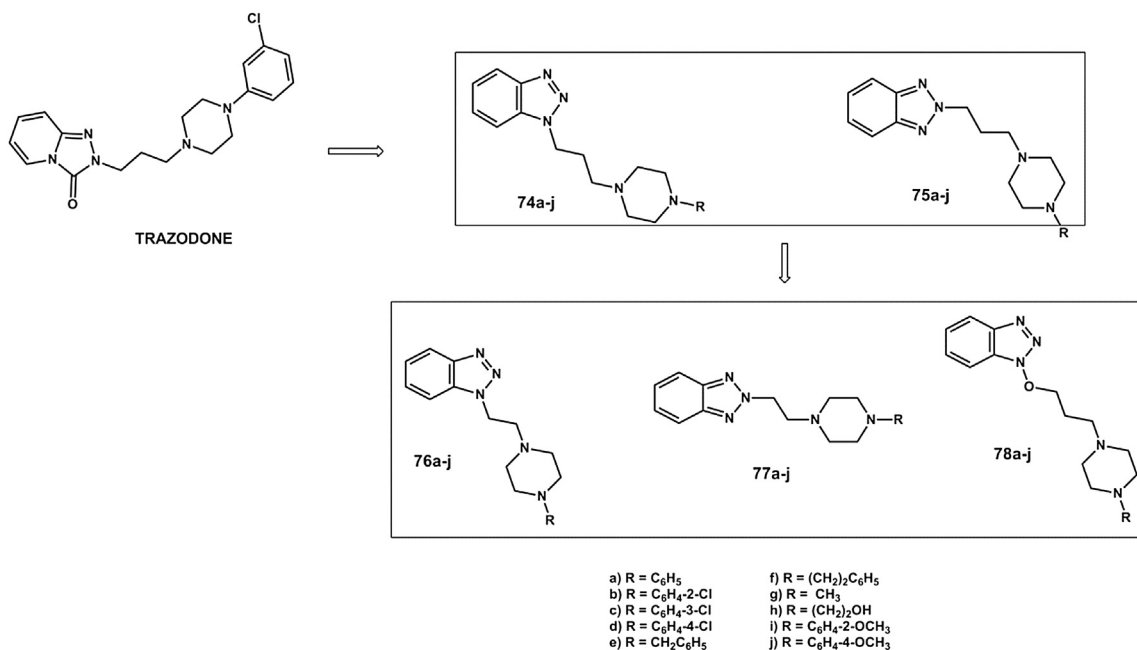


Fig. 53. Trazodone-like derivatives bearing a benzotriazole moiety.

For this reason, the research for new molecules able to selectively target tumor cells is still active. Particularly, nitrogen heterocycles, attracted the attention of researchers as possible isosteres of structural components of natural nucleotides [139–141], and also BT has been reported as possible anti-proliferative agent [107,142,143].

This principle of isosterism was followed by Zhan and Lou [144] to prepare new nucleoside analogues endowed with antitumor activity. Previously it was reported that carbocyclic nucleosides

(obtained by replacing oxygen with a sugar-CH<sub>2</sub> group) were more stable to the enzymatic action of nucleoside phosphorylase and they were also equipped with anti-tumor activity [145–147]. On this basis, Zhan and Lou decided to further modify the nucleoside analogues structure, replacing the heterocycle base with various polynitrogen heterocycles. With this aim azole nucleoside analogues of D-pinitol (=3-O-methyl-D-chiro-inositol) (**47a–d**) were synthesized passing through the key intermediate 3-O-methyl-4,5-epoxy-D-chiro-inositol (**46**). The different analogues of D-pinitol

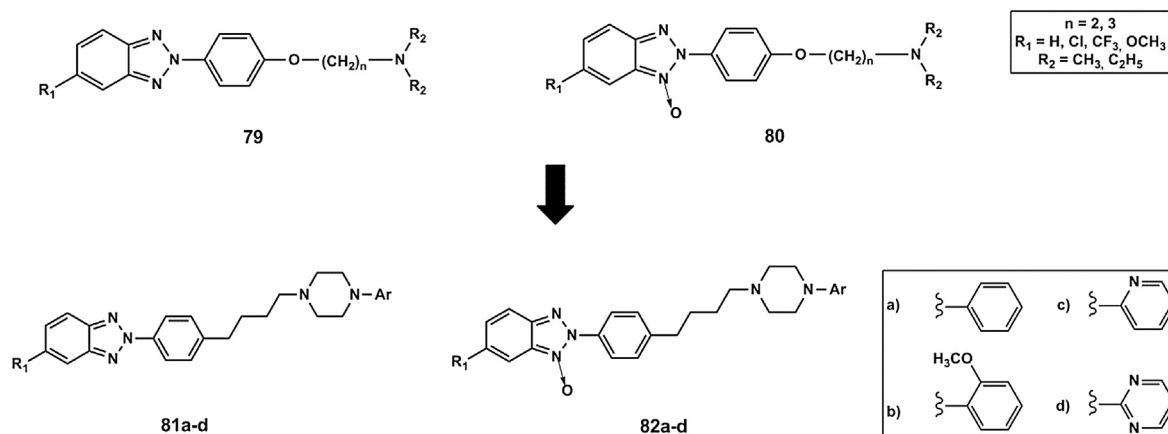


Fig. 54. Piperazinyl-phenyl-2*H*-benzotriazoles derivatives and their N-oxides.

were tested on tumor cell lines derived from human lung and bladder, but only derivatives with triazole (**46a**) and benzotriazole (**46b**) substitution were active, albeit at micromolar level, as shown in Fig. 34.

Al-Soud's research group worked following the same principle. They combined the potential activity reported for several alkylated benzotriazoles with that of 1,2,4 triazole nuclei (Fig. 35). Starting from 1*H*-benzotriazole-1 (**48a**) or 2 (**48b**)-carbonitrile were prepared azepinic compounds, which were found to be extremely promising after biological evaluation. From the analyzes performed on several human tumor cell lines, compound (**49a**) stood out for its micromolar activity against 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) tumor cells, although not showing selectivity in its action. In contrast, the N-2 isomer (**49b**) resulted inactive [148].

The interest generated by the indoloquinoline alkaloids, i.e. Cryptosanguinolentine [149,150], pushed the group coordinated by Beauchard to design and prepare a series of derivatives where the thiazole ring system is fused in a similar polycyclic system [151]. They prepared a series of new N-arylated thiazolobenzotriazoles as synthesis intermediates, all of them evaluated as potential anticancer agents [152]. Starting from 2-cyano thiazole-benzotriazoles (**50**) [29], compounds (**51a–h**) were prepared performing as substitution in nitrogen N-1 and N-3 with quinoline and pyridine nuclei. The reactivity of the cyano group at benzothiazole 2-position was exploited in order to prepare imidazoline derivatives (**52a–d**), obtained by reaction with ethylenediamine, as shown in Fig. 36.

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  $32.4 \pm 1.0$  for **51a** derivative, and  $97.4 \pm 1.0$  for **51f** on MDA-MB- 231, the most resistant cell line) with the exception of compounds (**51c**) and (**52c**), inactive on both lines. Generally the introduction of an imidazoline side chain determined an increase of the biological activity, with the exception of compound (**52d**).

Finally, the group of Wan and co-workers evaluated the antiproliferative activity of new benzotriazole derivatives [153–155]. The 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 derived from different tumor types. This molecule was therefore used in order to define a possible mechanism of action. The analyses carried out on BEL-7402 hepatocellular

carcinoma cells, the most susceptible to BmOB action ( $IC_{50} = 0.082 \pm 0.008$  mM), showed that this compound led to cell death through induction of a collapse of mitochondrial membrane potential, determining both the production of reactive oxygen species and DNA fragmentation [156].

Exploiting the concept of bioisosterism, on benzotriazolylpropiofenone derivatives [157,158] were introduced bulky side chains by reaction with nicotinic or isonicotinic acid. Only nicotinate showed some antitumor activity, and compounds (**53a,b** in Fig. 37) demonstrated potent propagation inhibition activity in liver and galactophore cancer cells [159].

Finally, as part of a wide study focused on benzotriazole derivatives that could act against *M. tb.*, Carta and coworkers disclosed that compounds belonging to the class of 3-Aryl-2-[1*H*-benzotriazol-1-yl]acrylonitriles (**54**) were able to inhibit cellular proliferation in a series of liquid and solid human tumors [14,105,106].

Principally, researchers identified (*E*)-2-(1*H*-benzo[d][1,2,3]triazol-1-yl)-3-(4-methoxyphenyl) acrylonitrile (**55**) as lead compound, shown in Fig. 38. This compound was 5–100 times more potent than 6-mercaptopurine and comparable to etoposide on all cell lines [107,142]. Similar derivatives bearing at benzotriazole positions 5 and 6 electron-donor substituents, such as methyl groups (**56**), resulted to be absolutely devoid of efficacy on the same tumor lines.

Therefore, in 2011 the group completed the SAR studies, introducing at position 5 and 6 electron-withdrawing groups. However, the synthesized derivatives (*E*)-2-(5,6-dichloro-1*H*-benzo[d][1,2,3]triazol-1-yl)-3-(4-R-phenyl)acrylonitriles (**57**) [142] resulted almost devoid of activity, demonstrating that the introduction of a chlorine atom in the benzotriazole moiety determined a considerable decrease of the antiproliferative activity for this class of compounds. Cell cycle analysis also revealed that this series acted during the G2/M phase of the cell cycle. This behavior was explained by a possible interaction of derivative with tubulin (cytoskeleton protein) and respective microtubules system [160,161].

This hypothesis was further confirmed by [<sup>3</sup>H]Colchicine competition-binding scintillation proximity assay (SPA) [162], in which one of the benzotriazole derivatives displaced 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*  $\beta$ -tubulin, which shows a 98% sequence homology with the human one

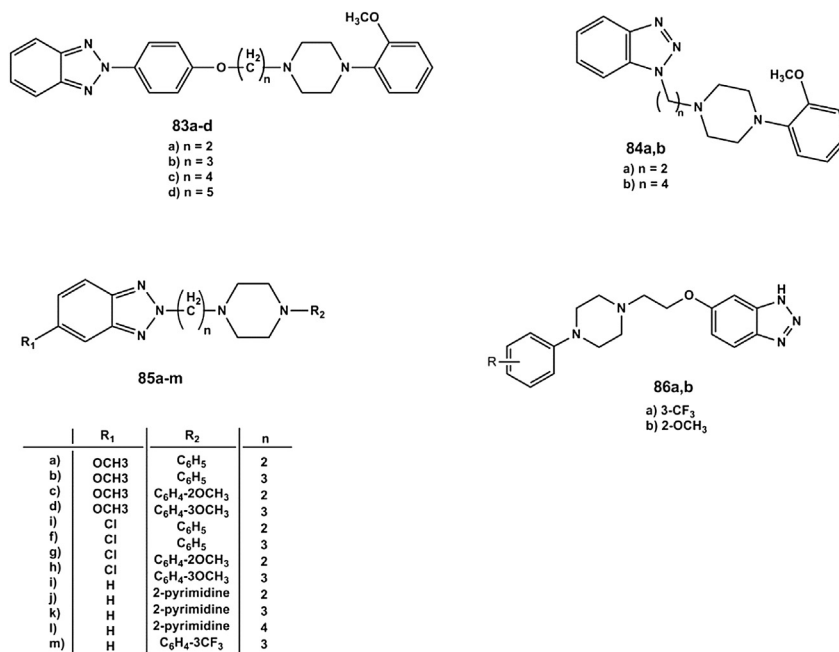


Fig. 55. Benzotriazole derivatives investigated as ligand for serotonin and dopamine receptors.

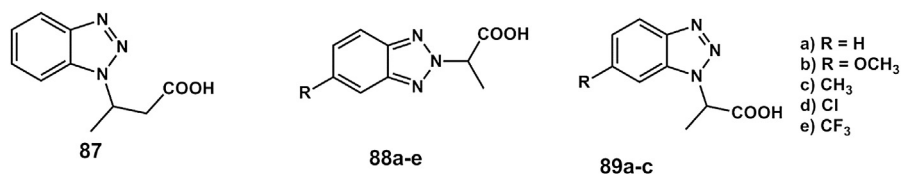


Fig. 56. 3-(Benzotriazol-1-yl)butanoic acid and (benzotriazol-1/2-yl)propionic acid derivatives.

(Fig. 39). This model 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 (**54**) and the amino acidic residues of the binding pocket.

From this analysis emerged that benzotriazole ring interacted with tubulin mainly through Van der Waals interactions and insertion of triazole moiety inside the colchicine binding site (Fig. 40). Consequently these compounds inhibited tubulin polymerization acting as microtubules destabilizing agents.

Howell et al. showed a preliminary study on a small set of 9-aminoacridine-3- and -4-carboxamides derivatives (**58a–d**), depicted in Fig. 41. All compounds were prepared using the benzyne/azide click chemistry and all of them were evaluated as possible DNA intercalators and antitumor agents. Unfortunately

none of the benzotriazole derivatives showed an appreciable displacement in an ethidium displacement assay at 2 μM concentration [163].

Finally, 1,3,4-oxadiazole derivatives containing benzotriazole moiety were prepared by Zhang and colleagues as potential anti-tumor agents. The biological target was identified in the focal adhesion kinase (FAK), a non-receptor tyrosine kinase that plays a leading role in cell proliferation. Among them compound (**59**, Fig. 42) stood out not only for its good antiproliferative activity against MCF-7 cells (IC<sub>50</sub> = 5.68 μg/ml), but also for the best FAK inhibitory activity (IC<sub>50</sub> = 1.2 μM, comparable to reference drug cisplatin). This lead compound was used to investigate its probable action mechanism in cell under apoptosis thought binding mode analyses on the target enzyme-active site.

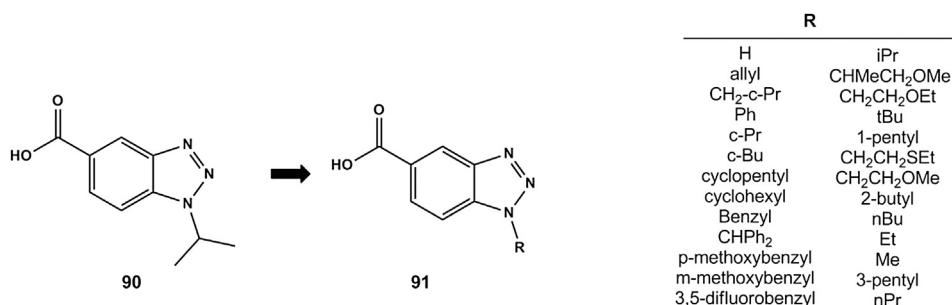


Fig. 57. 1-substituted benzotriazole-5-carboxylic acids identified by high-throughput screening as agonist of GPR109b.

## 8. 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 [164] involved in numerous metabolic processes. It is believed that the CK2 is involved in cell growth, both in healthy and cancerous cells [165], in the synthesis of tRNA and mRNA [166], as well as in apoptosis and in cell transformation. The CK2 has been described for the first time by Burnett and Kennedy [167] and different isoforms were identified [168,169]. However, the CK2 conventionally exists either in form of tetrameric complexes, composed of two catalytic subunits ( $\alpha$  and  $\alpha'$ , or  $\alpha''$ ) [170–172] and two regulatory subunits ( $\beta$  and  $\beta'$ ), or as free subunits [173,174]. There are also indications that tetrameric CK2 can be assembled into more complex structures. There are increasing evidences to suggest that different molecular forms of CK2 may be involved in different cellular activities. For instance, some substrates of CK2 can only be phosphorylated by tetrameric CK2, while others are phosphorylated exclusively by the free catalytic subunits [175]. Also, proteins able to interact with CK2 have been identified, and some of these discriminate between tetrameric CK2 and individual subunits [176].

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 [177]. More papers shown that evolution of neoplastic disease and cancer onset are directly proportional to the CK2 activity [178]. Furthermore, it was demonstrated that the CK2 shows oncogenic activity in transgenic mice and is frequently over-expressed in tumor and leukemia cells [179–181]. Finally, the high constitutive activity of CK2 is suspected to contribute to its pathogenic potential, since it is exploited by several viruses to exert the phosphorylation of proteins essential to their life cycle [182]. Furthermore, the catalytic subunit  $\alpha$  cooperates with other protooncogenes to promote cell transformation in different experimental models [179]. This makes CK2

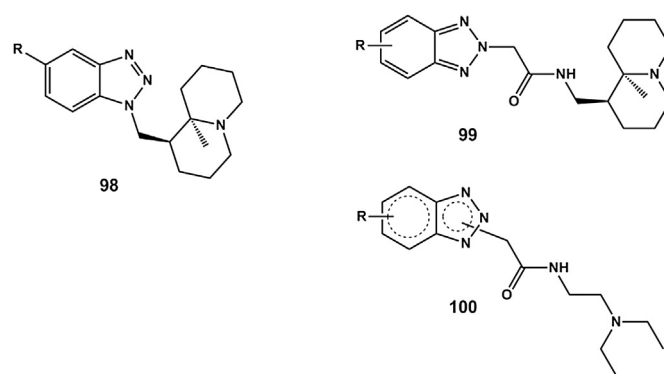


Fig. 59. Lupinyl and (tert-amino)ethyl benzotriazole derivatives.

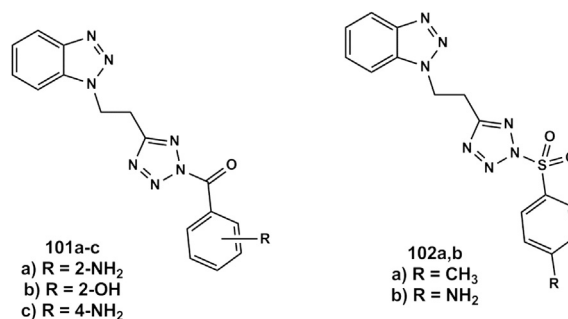
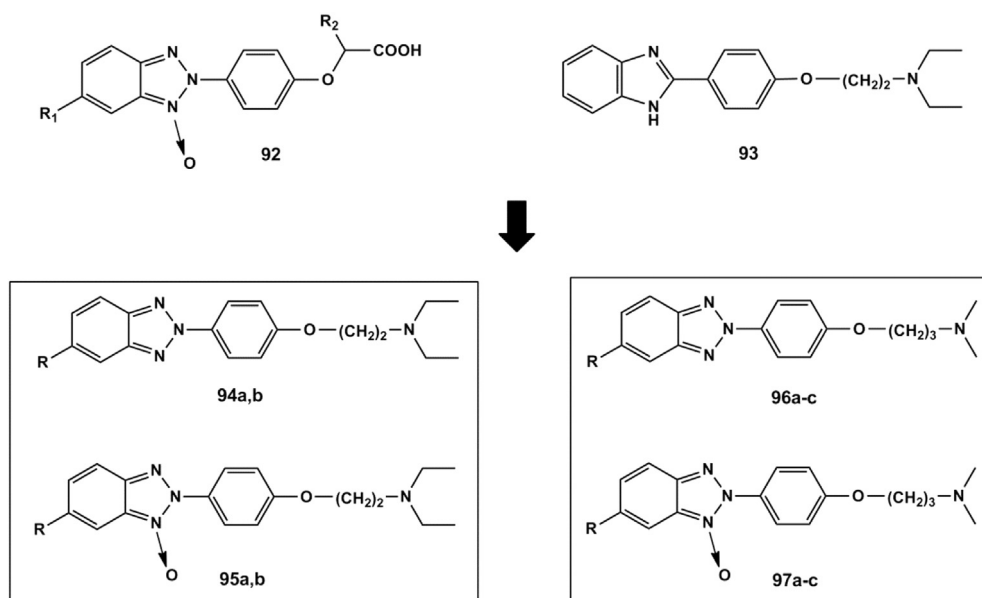


Fig. 60. Tetrazolyl-benzotriazole derivatives endowed with anti-nociceptive and anti-inflammatory activity.

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 4,5,6,7-tetrabromobenzotriazole (**60**,



R = a) H, b) Cl, c) CF<sub>3</sub>

Fig. 58. Series of 4'-(benzotriazol-2-yl)-phenylalkanoic and -phenoxyalkanoic acids and derivatives.

TBBt) and 4,5,6,7-tetrabromobenzimidazole (**61**, TBBi), shown in Fig. 43.

Sarno and co-workers showed that TBBt is the most selective and specific CK2 inhibitor so far analysed. Among the 33 protein kinases tested by this research group, only three were inhibited by TBBt and their  $IC_{50}$  values appeared to be about two orders of magnitude higher than the values calculated using CK2. The three kinases inhibited by TBBt were CDK2 and GSK3L, belonging to the same protein kinase subfamily (named CMGC group), and PHK, belonging to the CaMK group. Moreover, within the same CK family, TBBt turns out to be more selective against CK2 ( $K_i = 0.4 \mu\text{M}$ ) in comparison with CK1 ( $K_i = 47 \mu\text{M}$ ) [183]. A third class of CK proteins, localized to the Golgi apparatus, was instead totally unresponsive to TBBt [184]. All this evidences suggested that TBBt possess a particular selectivity only towards CK2 as inhibitor agent. This behavior, combined with the molecular ability to penetrate inside the cell and the lack of evident short term cytotoxicity made the TBBt a promising lead to design new compounds with high therapeutic potential.

Zien research group compared the activity of TBBt and TBBi toward CK2 purified holoenzymes, showing that the latter was not only far more effective in discriminating between different forms of CK2 present in yeast [185,186], but also dramatically more effective in the induction of apoptosis and, to a lesser extent, necrosis in transformed human cells [187]. Moreover, the solution of the complex CK2/TBBi crystal structure [188] allowed a comparison with the analogue CK2/TBBt complex [189], leading to the conclusion that despite their structural similarities these molecules bound the biological target in a quite different manner.

The history of TBBt born in 1950, when it was demonstrated that the 1- $\alpha$ -ribofuranosido-5,6-dimethylbenzimidazole (**62**, Fig. 44) represented a key constituent for vitamin B<sub>12</sub> [190,191]. This observation stimulated the synthesis of various structural analogues to evaluate their biological activity, particularly on cell proliferation. It was discovered that the 5,6-dichloro-1-( $\beta$ -D-ribofuranosyl)benzimidazole (**63**, DRB) was able to inhibit *in vitro* and *in vivo* mRNA synthesis in eukaryotic cells [192]. Additionally, 5,6-dibromo-1- $\beta$ -D-ribofuranosylbenzimidazole (**64**) demonstrated to be a potent inhibitor of *in vitro* and *in vivo* transcription, concomitantly inhibiting purified CK2 activity at 6–10 times lower concentrations than DRB [193].

All these works motivated the studies on other halogenated benzimidazole nucleoside analogues [175,194] and led eventually to the synthesis of TBBt [63]. The inhibitor specificity of TBBt was then exploited extensively to understand the functionality of the CK2. The research group of Battistutta, for instance, studied the

interactions between TBBt and *Zea mays* protein kinase CK2 $\alpha$  catalytic subunit. Particularly, they delineated the enzyme–inhibitor complex crystal structure. The specificity and selectivity of TBBt for CK2 appeared to be mainly directed by the reduced size of the active site, a small hydrophobic pocket adjacent to the ATP/GTP binding site, where TBBt perfectly fit, as depicted in Fig. 45.

The main interactions between TBBt and CK2 were hydrophobic in nature and involved the four bromine atoms. The halogen size allowed the TBBt perfect insertion into the cavity and, concomitantly, the release of the inhibitor, once it has been trapped inside the pocket [189].

The inhibitory power decreased if the TBBt four bromine atoms were replaced with smaller atoms, for instance chlorine atoms, as previously observed by the Szyszka's research group [63]. Therefore, TBBt lose its inhibitory potency against the majority of protein kinases because in most other protein kinases the hydrophobic binding site is generally too large to generate stable interactions with the inhibitor.

The relevance of the individual bromine atoms of TBBt was then investigated by the group of Wasik, which synthesized all the possible TBBt isomers. Mono-, four di-, and two tri-bromobenzotriazoles were prepared and their physico-chemical properties were evaluated in an aqueous medium. The analysis confirmed that hydrophobic and electrostatic interactions were predominant in fully halogenated benzotriazoles, determining a selective inhibition of protein kinase CK2 $\alpha$  [195].

In order to increase the TBBt and TBBi activity as CK2 inhibitors, the research groups of Bretner and Najda-Bernatowicz designed new tetrabromine derivatives. They evaluated the influence on TBBt/TBBi inhibitory potency of TBBt/TBBi derivatives bearing alkyl substituents with different hydrophobic, steric and electrophilic features. Their research program was based on the evidence that water molecules, necessary for enzymatic activity, were coordinated at the binding site on CK2 [188], molecules that could be displaced by similar substituents. New N-hydroxyalkyl derivatives

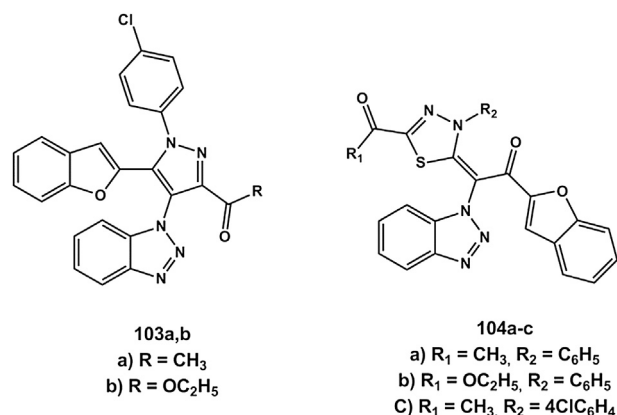


Fig. 61. Benzotriazole derivatives bearing benzofuran nucleus.

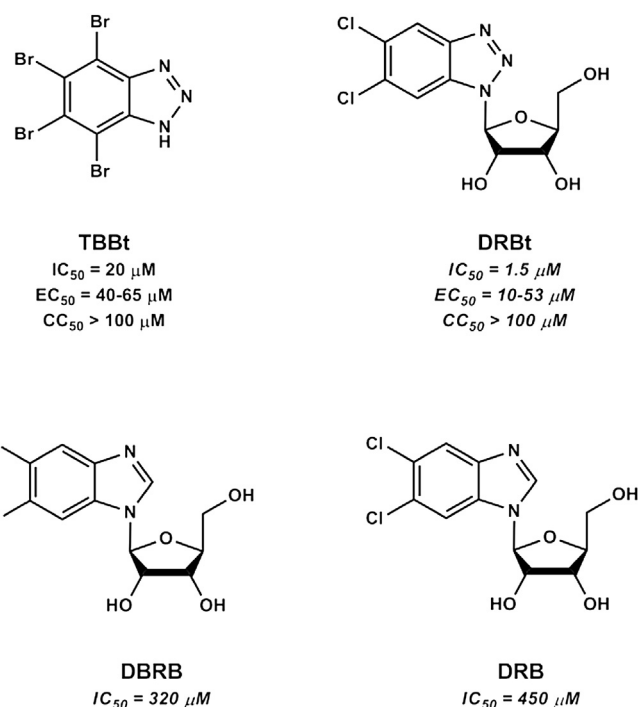


Fig. 62. Structures and anti HCV (DNA substrate) activity of benzimidazole and benzotriazole derivatives.

(65) were then tested on human CK2 holoenzyme ( $\alpha_2\beta_2$ ). Evidences demonstrated that the pharmacological activity depended on alkyl chain length. Some of the N-hydroxyalkyl derivatives showed  $IC_{50}$  values similar to that of the parent compounds, and best results were ensured by the propan-1-ol chain, with  $IC_{50}$  values of  $0.48 \mu\text{M}$  [196]. 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-1H-benzimidazol-1-yl)propan-1-ol (**65a**) appears to be the best of the series, with small differences from the N2-substituted derivative (**65b**) (Fig. 46). On the contrary, for derivatives bearing shorter or longer alkyl chains a drastic decrease in their activity was observed. Additionally they demonstrated that the four bromine atoms in the benzene ring seemed to be an essential requirement for biological activity. Indeed, the substitution of bromine with chlorine or  $\text{CH}_3$  groups leads to significantly lower inhibitory effect versus different forms of CK2, human and not. The relevance of the triazole system was finally underlined by its replacement with a phthalimide moiety, feature present in many biologically active compounds [197,198], or with a phthalazine system. Indeed, the derivatives thus obtained resulted powerless [199].

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 [200], especially at the light of the biological successes reported by larger molecules [201,202].

New researches led to derivatives bearing on the triazole moiety even bulkier side chains. A modification of the tetrabrominate benzene ring was also evaluated and the two bromine atoms in 4 and 5 were replaced with aryl and alkyl substituents. Unfortunately, in comparison with TBBt ( $IC_{50} = 0.46 \text{ mM}$ ) no increase in activity was observed for most of the compounds, except for 5,6,7-tribromo-4-methyl-1H-benzotriazole (**66**,  $IC_{50} = 0.51 \mu\text{M}$ ) and 5,6,7-tribromo-4-ethyl-1H-benzotriazole (**67**,  $IC_{50} = 0.16 \mu\text{M}$ ), as shown in Fig. 47 [203].

The Swider group synthesized new benzotriazole derivatives using a receptor-based design approach to obtain compounds able to interact simultaneously with the  $\text{Mg}^{2+}$ -chelating residues and

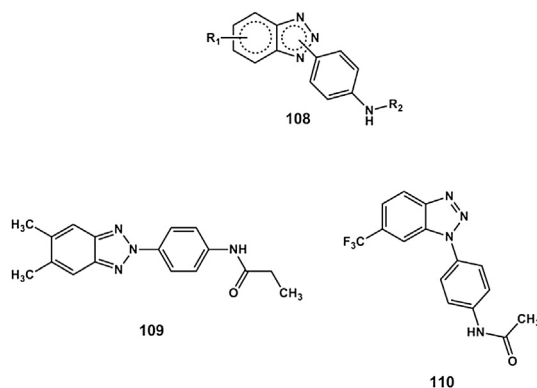


Fig. 64. N-[4-(1H(2H)-benzotriazol-1(2)-yl)phenyl]alkylcarboxamides derivatives.

the protein-substrate binding residues [204]. These compounds were obtained by Azide-alkyne Huisgen cycloaddition on tetrabromobenzotriazole derivatives. The result was a combination of the tetra-halogenated moiety, which nicely occupied the ATP-binding site, with side chains able to bind simultaneously both enzymatic active site and basic residues that participate in protein substrate binding, like similar bisubstrate inhibitors of other kinases [205]. In this paper were reported the preliminary and non-optimal results for the most active compound (**68**,  $IC_{50} \mu\text{M}$   $6.33 \pm 0.23$ ), which synthesis is reported in Fig. 48.

Finally, it is important to report the new synthetic approach of Wawro and co-workers, which was based on two considerations: first, none of the previously synthesized halogenated derivatives of TBBt had a chiral center, and second, 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 [199] and Makowska [203], the group developed a new synthetic approach to isolate enantiopure hydroxyalkyl derivatives of TBBt (**69a–c**, Fig. 49), obtaining optically active compounds endowed with enantioselectivity factor ( $E > 200$ )

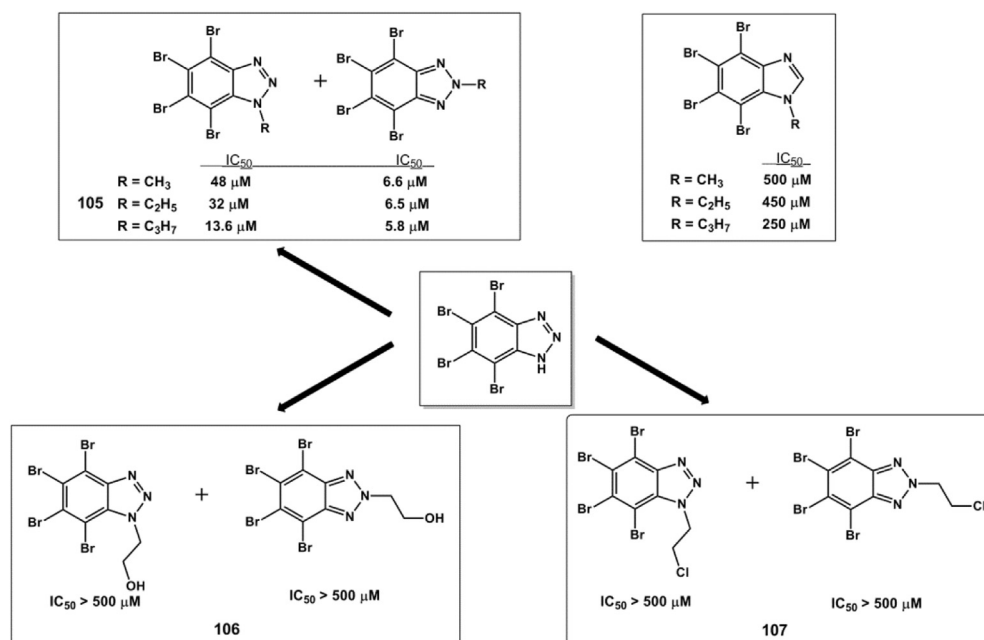


Fig. 63. Structures and anti-HCV helicase activities of 1H-tetrabromobenzotriazole and tetrabromobenzimidazole derivatives using a DNA substrate.

[206]. These molecules, potentially CK2 inhibitors, might be used to synthesize new optically pure TBBT derivatives.

### 9. Benzotriazole in coordination compounds as antitumor agents

Benzotriazoles can form complexes with different transition metals [207] and a study on *N*-(4,5-dihydroimidazol-2-yl)azoles derivatives involved in coordination complexes with transition metals highlighted their potential anticancer properties [208]. On this basis, the group of Saczewski reported the synthesis and biological evaluation of some chelating bidentate 2-substituted benzotriazole copper (II) complexes [209]. They also designed new molecules with the idea to create compounds endowed with potential copper–zinc-superoxide dismutase (Cu, Zn-SOD) mimicking activity. According to the theory of Oberley and Buettner, SOD activity in tumor cells is lower than that found in normal cells [210]. (70a) and (70b) complexes showed 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 was exquisitely sensitive to the structure of the coordinating ligand (Fig. 50). The derivative of substitution in 2 showed a potent *in vitro* SOD activity, with 0.06  $\mu\text{M}$  IC<sub>50</sub> value, comparable with data reported in literature for other mimicking SOD agents. Evaluating the *in vitro* cytotoxicity on seven tumor lines, once again the derivative of substitution in 2 turned out to be the best one, recording an IC<sub>50</sub> values between 13 and 28  $\mu\text{M}$ .

### 10. Benzotriazole as histone deacetylase (HDAC) inhibitor

The histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl groups from lysine residues, especially in histones H3 and H4 [211]. This chemical modification is a key step in the genes expression regulation which influence cell differentiation and proliferation processes [212]. An abnormal HDACs functioning was linked to the carcinogenesis process [213]. In fact, many HDACs inhibitors can exert *in vivo* an antitumor effect [214].

There are 18 humans HDACs generally divided into four classes, according to their homologies to yeast's HDACs, 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 [215]. HDAC inhibitors can be classified into five groups, according to their chemical structure: 1) hydroxamic acids, 2) cyclic tetrapeptides (apicidin); 3) short chain carboxylic acids, such as valproic acid; 4) benzamides 5) ketoacids [216].

However, in a recent paper by Fu and co-workers, even benzotriazole derivatives were recognized as antiproliferative agents that exert their biological action through HDAC inhibition [217]. The group synthesized various benzotriazole compounds bearing substituted benzoic acids. Their antitumor activity was evaluated on three different human tumor cell lines. Compounds showed moderate antiproliferative activity with the exception for 1*H*-Benzo[d][1,2,3]triazol-1-yl 3,4,5-trimethoxybenzoate (71). This compound stood out with an IC<sub>50</sub> value of 1.2–2.4 nM, very close to that of positive control doxorubicin. The SAR analysis emphasized the relevance of the OCH<sub>3</sub> group for the antiproliferative activity. In fact, derivatives bearing such substituents at benzoic ring positions 3, 4 and 5 were the most powerful, while methoxyl substitution of benzotriazole group resulted in a drastic reduction of antiproliferative activity [217]. 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. Analyzing quantitative experimental results obtained it was clear that the more potent derivative was once again the trimethoxy derivative, in agreement with the antiproliferative activity results. This derivative was finally subjected to molecular docking studies to simulate its interactions with the HDAC using the AUTODOCK 4.0 software [218]. The analysis highlighted the hydrophobic interactions between the benzotriazole and phenyl moieties and Phe141, Tyr196, Leu265, Lys267, Tyr297 of the enzymatic binding pocket (Fig. 51). Three dimensional quantitative structure–activity relationship (3D-QSAR) and additional molecular docking studies on this class of compounds were performed to obtain informations for rational design of novel benzotriazoles endowed with more potent antiproliferative activity [219].

### 11. Benzotriazole alkanolic acids derivatives as peroxisome proliferator-activated receptors (PPARs) agonist

The peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of intracellular receptors and regulate many biological processes, including energy metabolism (glucose and lipid metabolism), cell proliferation, skin development and inflammatory process [220,221]. The PPAR $\gamma$  is a member of the steroid nuclear receptors superfamily second class and regulates gene transcription through the formation of functional heterodimers with retinoid X receptor (RXR) for 9-*cis*-retinoic acid. The PPAR/RXR complex modulates the expression of target genes by recognizing specific responsive sequences consisting of a direct repeat (DR) of a hexanucleotide sequence [5'-TGA(A/C/T)CT] separated by a single nucleotide (DR1) [222].

The PPAR subfamily includes three isoforms:  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$ . Encoded by different genes, only for the PPAR $\alpha$  and PPAR $\gamma$  were identified specific ligands, consisting on saturated and

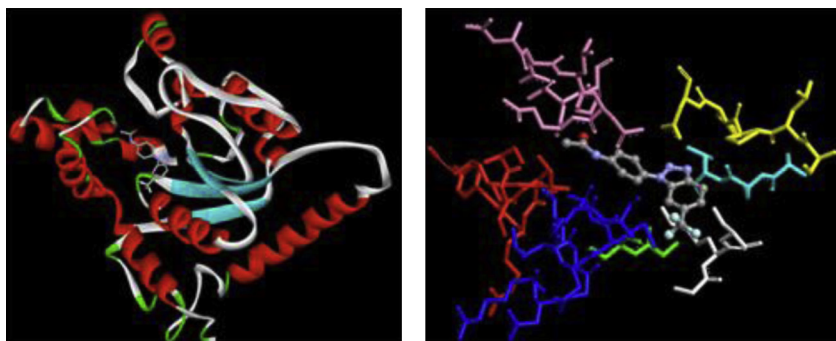


Fig. 65. Cartoon of the binding mode of compound **n** in the putative binding site of the Polio (Sb-1) helicase and snapshot of the docked compound in the putative binding site.



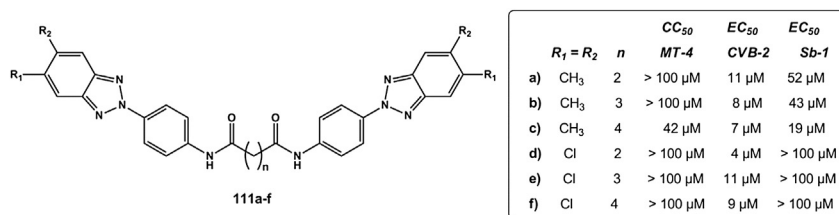


Fig. 66. *N,N*-bis[4-(2*H*)-benzotriazol-(2-yl)phenyl]alkylcarboxamides and their antiviral activities.

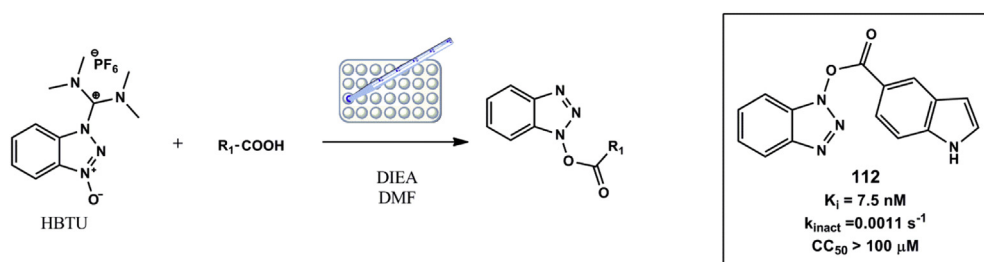


Fig. 67. Synthesis of benzotriazole esters starting from HBTU and 90 acids in microtiter plates, and 1*H*-benzo[*d*][1,2,3]triazol-1-yl 1*H*-indole-5-carboxylate activity.

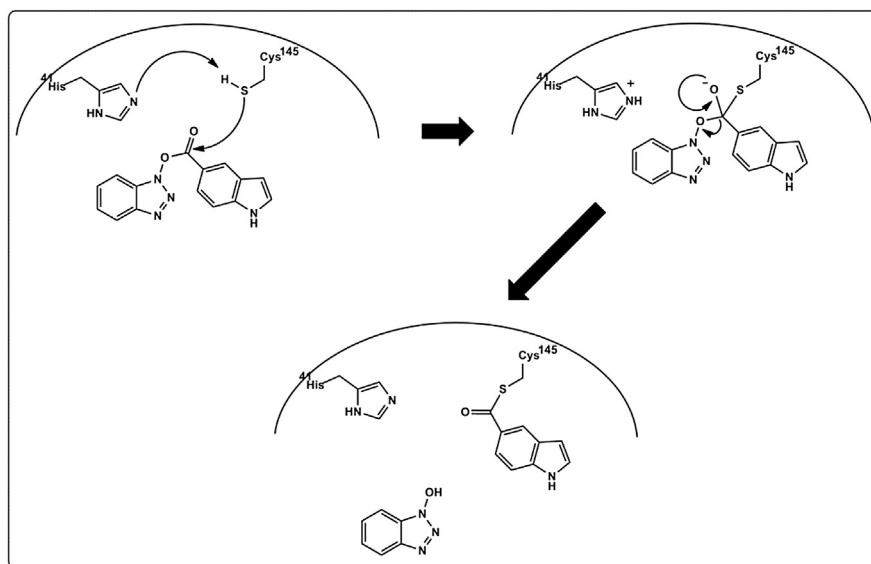


Fig. 68. Proposed mechanism for inhibition of SARS-CoV 3CL<sup>pro</sup> by acylation with benzotriazole esters by Wu et al.

polyunsaturated long-chain fatty acids, eicosanoids, and hypolipidemic agents. In particular, among PPAR $\alpha$  agonists we can remember the fenofibrates, gemfibrozil, bezafibrate and clofibrate, all developed as drugs for the treatment of dyslipidemia. Pioglitazone and rosiglitazone are agonists of PPAR $\gamma$  and were developed as drugs for the treatment of type 2 diabetes mellitus [223]. 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 [224,225].

According to studies conducted by the Sparatore's 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) a hydrophobic tail (formed by a mono- or polycyclic aromatic or heteroaromatic moiety), as in rosiglitazone (Fig. 52).

Thus, to develop new PPAR  $\alpha$ ,  $\beta/\delta$ , and  $\gamma$  activators, Sparatore et al. decided to stiffen the last two parts and suppressed the flexible chain linker between the aromatic rings. A new series of [4-(2*H*-1,2,3-benzotriazol-2-yl)phenoxy]alkanoic acids (**73**) was synthesized [226]. The 4-(2*H*-1,2,3-benzotriazol-2-yl)phenoxy moiety, which characterized these compounds, was also present in the 5-chloro-2-(4-[3-(dimethylamino)propoxy]phenyl)-2*H*-1,2,3-benzotriazole (**72**). The latter was recognized as able to significantly reduce the cholesterol level in hypercholesterolemic mice, measurements comparable to that of bezafibrate [227]. Undergoing biological tests, the study showed that the compounds 3-(4-(2*H*-benzo[*d*][1,2,3]triazol-2-yl)phenoxy)-2,2-dimethylpropanoic acid (**73a**) and 3-(4-(2*H*-benzo[*d*][1,2,3]triazol-2-yl)-2,6-dimethylphenoxy)-2,2-dimethylpropanoic acid (**73b**), dual activators of PPAR  $\alpha/\delta$  and PPAR  $\alpha/\gamma$ , respectively, possessed an effectiveness equal to that of the reference compound Wy-14643 [228], but differed strongly in potency, presenting respectively  $EC_{50}$  of

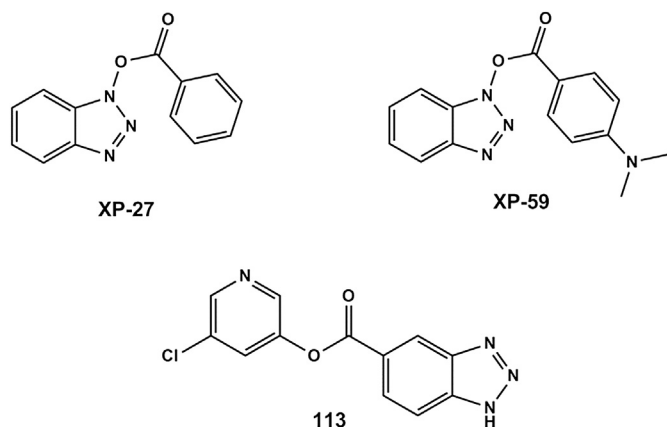


Fig. 69. SARS-CoV proteinase inhibitors.

10.5  $\mu\text{M}$  and 96 nM on Human PPAR  $\alpha$  ( $\text{EC}_{50}$  Wy-14643 = 9.3  $\mu\text{M}$ ) (Fig. 52).

Interestingly, 5-chloro-2-(4-[3-(dimethylamino)propoxy]phenyl)-2H-1,2,3-benzotriazole (**72**) was found to be inactive on all three receptors at concentration up to 100  $\mu\text{M}$ , demonstrating that probably the lipid-lowering effect of these molecules was due to a different mechanism of action. In summary, the obtained results showed that the 4-(2H-1,2,3-benzotriazol-2-yl)phenoxy moiety, which characterized these compounds, represented 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.

## 12. 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 several physiological functions, including appetite control, mood regulation, voluntary movement, circadian cycle, body temperature, attention, memory and learning [229–231]. Alteration in the serotonergic system may induce psychiatric disorders such as anxiety, depression and obsessive-compulsive disorder [232]. For this reason, in recent years, dopamine and serotonin agonists and antagonists have been administered for the treatment of schizophrenia and Parkinson's diseases [233–235].

The Caliendo's research group reported a series of 1/2-[3-(4-(R)-1-piperazinyl)alkyl]-benzotriazole derivatives, designed as structural analogues of trazodone, known psychoactive drug belonging to the piperazine and triazolopyridine class. Synthesized derivatives (Fig. 53) contained three major structures: 1) an N-4 substituted piperazine ring; 2) a cyclic system containing an unsubstituted benzotriazole nucleus; 3) an alkyl or alkyloxy linker which acts as a linker between the piperazine and benzotriazole moiety.

First series of 1- and 2-[3-(4-(R)-1-piperazinyl)-propyl]benzotriazoles (**74,75a–h**) was prepared and all derivatives were screened *in vitro* for anti-serotonin, anti-adrenergic and antihistaminergic effects, as well as *in vivo* for analgesic activity. Concerning the anti-serotonin activity, results showed that 1-benzotriazole derivatives were more powerful than 2-substituted isomers, and compound (**75a**), bearing on the 4-piperazine nitrogen an unsubstituted phenyl ring, stood out for its potency [236].

With the aim of broadening the structure–activity relationships (SAR) for this class of analogues, the group synthesized a new series of compounds shortening or modifying the alkyl bridge between the benzotriazole ring and the piperazine nitrogen. Particularly, they replaced the propylene linker with an ethylene or an

oxypropylene moiety. Sets of 1- and 2-[2-[4-(R)-1-piperazinyl]ethyl]benzotriazoles (**76,77a–h**) and a set of 1-[3-[4-(R)-1-piperazinyl]propoxy]benzotriazoles (**78a–h**) were prepared and screened [237]. Once again, the most powerful derivatives belonged to the 1-(2-(4-(R)-1-piperazinyl)-ethyl)-benzotriazoles series (**76a–j**). Derivatives bearing alkyl substituents on the 4-piperazine nitrogen (**76g,h**) showed none or extremely low activity. Moreover, the introduction of a chlorine atom at the ortho (**76b**) and meta (**76c**) positions of the phenyl ring determined an increase of activity. On the contrary substitution at the para position (**76d**) determined a loss of it by 10–100 times. Still, the introduction of a methylene (**76–78e**) or ethylene (**76–78f**) bridge between the aromatic ring and the piperazine nucleus led to an activity decrease due to steric and electronic effects, rather than hydrophobic effect.

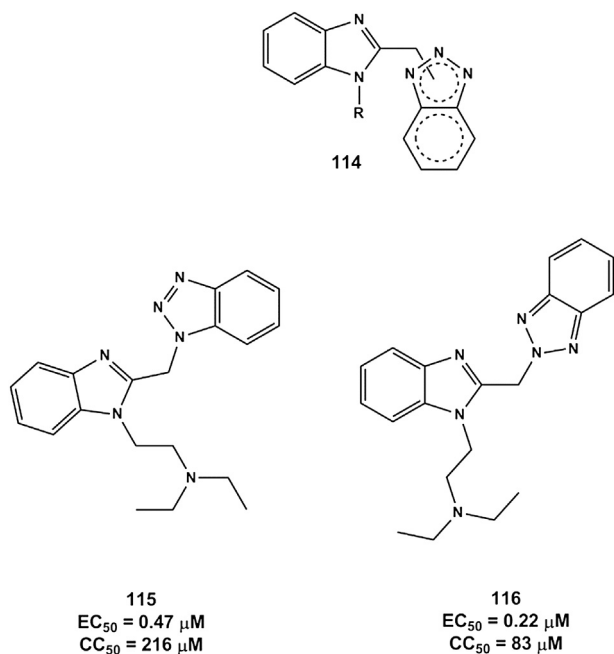
The *in vivo* noradrenaline-induced vas deferent contraction inhibition was investigated to determine the anti-adrenergic activity. All derivatives of substitution on benzotriazole N1 and N2 bearing on piperidine moiety a phenyl or a chlorophenyl (**74–77a–d**) resulted actives.

The anti-histamine effect was instead determined for compounds **78a–e** bearing simultaneously as side chains an oxypropylene bridge and an aromatic moiety. The substitution of the aryl or benzyl group with a methyl or a  $\beta$ -hydroxy-ethyl chain led to significant activity reduction.

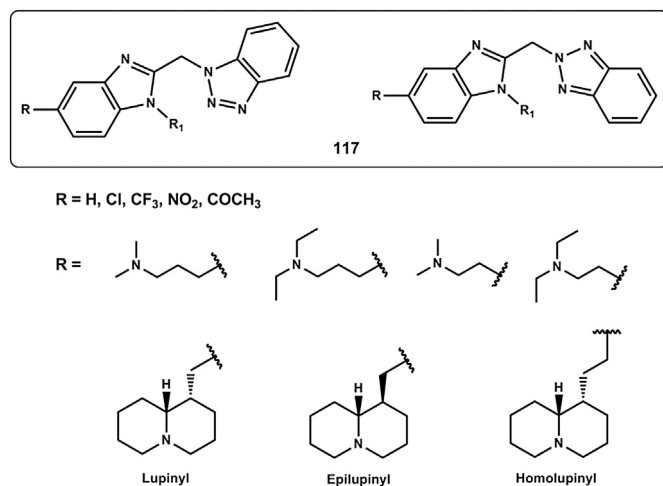
In 1996, the Caliendo group, through binding assays with radiolabeled ligand assays, highlighted the affinity of previously synthesized derivatives (**74–78a–h**) and newly synthesized compounds (**74–78i,j**) for the recombinant human receptor subtypes 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>1D $\beta$</sub>  [238]. The experimental results showed that most of compounds possessed a high affinity for the 5-HT<sub>2A</sub> receptor, while none of them bore affinity for subtype 5-HT<sub>1D $\beta$</sub> . The bond to the 5-HT<sub>2A</sub> receptor was favored by groups Ph, 3-Cl-Ph or 4-Cl-Ph on the N-4 piperazine ring. Instead, about 5-HT<sub>1A</sub> receptor, the potency increased significantly when a substituent 2-CH<sub>3</sub>O-Ph was located at the N-4 piperazine ring. Finally, authors deduced that the different receptors binding affinity was due to the double effect of the R substituent and the type of carbon linker. These evidences were justified through a different modes of interaction between ligand and receptor, as postulated by Ismael et al. [156] The Caliendo's research group also compared the binding affinity and selectivity of derivatives for different receptor isoforms, expressing it as 5-HT<sub>2A</sub>/5-HT<sub>2C</sub> ratio. The best selectivity ratio was obtained when R was a Ph or a 4-Cl-Ph group. On the contrary, when R was a 2-CH<sub>3</sub>O-Ph group the derivative acted as a 5-HT<sub>1A</sub> antagonist [238].

Other potential receptor ligands and trazodone like compounds were those reported by Sparatore et al., whom analyzed the benzotriazole pharmaceutical properties for long time. The group prepared a series of 2-[4-[3-(4-aryl/heteroaryl-1-piperazinyl)propoxy]phenyl]-2H-benzotriazoles (**81a–d**) and their N-oxides (**82a–d**) [239]. Their design was based on previous biological evidences reported for a series of 2-4-(dialkylaminoalkoxy)phenyl]benzotriazoles (**79**) and corresponding N-oxides (**80**) [227]. All series are depicted in Fig. 54.

Indeed, the aryl/heteroaryl moiety is a well-known ligand for 5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptors, but the affinity of these derivatives for dopamine and adrenergic  $\alpha_1$  receptors has not been excluded [240,241]. Compounds (**81a–d**) and (**82a–d**) were able to bind dopamine receptors D<sub>2</sub> and D<sub>3</sub> as well as serotonin receptors 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub>, however they presented low affinity for subtypes 5-HT<sub>1B</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>3</sub>, and 5-HT<sub>4</sub>. Particularly compound (**82b**), containing the 2-methoxyphenyl residue and the N-oxide function, was definitively the most active ( $K_{i5\text{-HT}_{1A}} = 11.9$  nM). On the contrary, the N-oxidation of benzotriazole ring exerted a negative influence on the affinity for 5-HT<sub>2A</sub> receptors.



**Fig. 70.** Lead compounds benzotriazole-derived that act as inhibitors of Respiratory Syncytial Virus fusion.



**Fig. 71.** 1-Substituted-2-[(Benzotriazol-1/2-yl)methyl]benzimidazoles proposed by Tonelli et al.

Also 2-(4-[ω-(4-(2-Methoxyphenyl)-1-piperazinyl)alkoxy]phenyl)-2H-benzotriazoles (**83a–d**) and their N-oxides showed affinity for 5-HT<sub>1A</sub> and D3 receptors. The affinity increased with the length of the aliphatic linker, and obtained derivatives having 4–5 methylenes bridge exhibited good or excellent selectivity for 5-HT<sub>1A</sub> and D<sub>3</sub> [242]. Finally, 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<sub>1A</sub> receptors [243].

Paluchowska and co-workers highlighted the relevance of the aliphatic linker length between methoxy phenyl piperazine moiety and the benzotriazol-1-yl nucleus (Fig. 55). They synthesized and evaluated a series of aryl piperazines in which the linker was composed of two to four methylene groups. Part of this set proved to act as a ligand of the 5-HT<sub>1A</sub> receptor, with K<sub>i</sub> between 4 and

88 nM. Derivatives with bi-methylene bridges resulted inactive *in vivo* (**84a**), while those with tetra-methylene bridges (**84b**) proved to operate as antagonists for postsynaptic 5-HT<sub>1A</sub> receptors [244].

More aryl/heteroaryl-piperazinyl alkyl benzotriazoles (Fig. 55) were prepared and evaluated, by modification of the benzotriazole moiety (introduction of substituents such as Cl and OCH<sub>3</sub>) or the aryl side chain (introduction of 2-pyrimidinyl or 3-trifluoromethylphenyl groups). The aim of these modifications was to increase the affinity of the derivatives to the 5-HT<sub>1A</sub> receptors. In fact, new compounds (**85a–m**) demonstrated moderate to good affinity for serotonin receptor, whereas none or modest affinity for dopamine D2 receptor [245]. Particularly, affinity of compounds (**85c,d,h,g,l**) was higher than trazodone and often comparable with buspirone.

Finally, to confirm the relevance of previously described structures, here is reported the failure of trifluoromethyl and methoxyphenyl piperazin-1-yl-ethoxy-1H-benzotriazoles derivatives (**86a,b**), depicted in Fig. 55, which came out completely inactive as 5HT<sub>1A</sub> and D2 competitor [246].

### 13. Choleric, cholesterol-lowering and anti-inflammatory activity of benzotriazole

In 1964 Sparatore group described a series of benzotriazolylalkanoic acids. The 3-(benzotriazol-1-yl)butanoic acid (**87**, Fig. 56), emerged for its strong choleric activity [247,248]. Therefore a series of structural changes were carried out in order to understand the meaning of modifications on the heterocycle aromatic moiety [249,250]. Results demonstrated that the introduction of a methyl or a methoxy group at benzotriazole 6 position caused a biological activity decrease in comparison with the 3-(benzotriazol-1-yl)butanoic acid, while the presence of a trifluoromethyl group increased it.

Side chain modification on benzotriazole nitrogen N-1 had also consequences: the activity was maintained introducing unsaturation into the N-alkyl chain, but was lost shortening it [25]. For some of these derivatives the antinociceptive and anti-inflammatory activity was evaluated, and 2-(benzotriazol-1/2-yl)propionic acids (**88a–e**) and (**89a–c**) exhibited significant activity as anti-inflammatory and antinociceptive agents [251].

A series of 1-substituted benzotriazole-5-carboxylic acids (**91**) (Fig. 57) was synthesized on the template of 1-isopropylbenzotriazole-5-carboxylic acid (**90**), identified by high-throughput screening as agonist of GPR109b [252], receptors predominantly expressed in human adipocytes which mediate antilipolytic effects [253]. All compounds biological activity was measured using a cAMP whole-cell assay and all of them acted as agonist for GPR109b, most with pEC<sub>50</sub> between 6 and 7. Significantly lower activity was reported for cycloalkyl-substituted derivatives (with the only exception for the cyclopentyl derivative). Additionally, these compounds showed an excellent selectivity for GPR109b vs GPR109a, and none of them determined the activation of homologous mouse receptor PUMA-G. Bioisosteric modification of carboxylic moiety with tetrazole motif was done to explore the limits of the binding pocket. None of these compounds had activity in GPR109b assay, suggesting that enlargement in the molecular acid region could not be tolerated.

Researchers synthesized many other alkanoyl acids, including a series of 4-(benzotriazol-1-oxide-2-yl) phenoxy alkanoyl acids and their N-oxides (**92**), endowed with different biological properties, such as anti-inflammatory, diuretic or anti-hypertensive [254,255]. Over the years additional modifications were done based on Buu-Hoi studies [256]. Some substituted benzimidazoles, such as 2-[4-(2-diethylaminoethoxy)phenyl]benzimidazole (**93**), exhibited

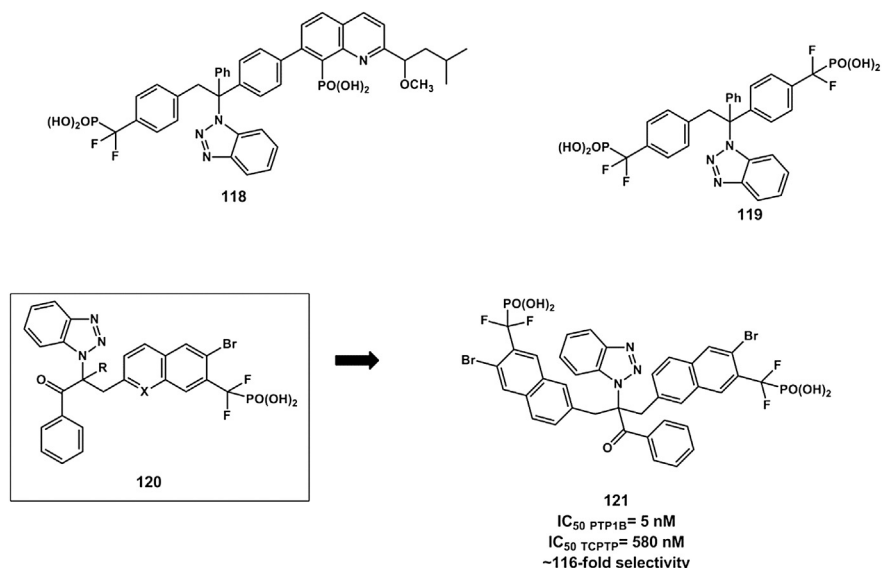


Fig. 72. Benzotriazole derivatives containing a difluoromethylphosphonates moiety as PTP1B inhibitors.

strongly depressive effects on the CNS and cholesterol biosynthesis inhibition. Basing on this evidences a series of 2-[4-(dialkylaminoalkoxy)phenyl]benzotriazoles (**94,96**) and their N-oxides (**95,97**) was synthesized [227], as described in Fig. 58:

The pharmacological investigation demonstrated that compounds **96c** and **97c** showed different behaviors:

- 1) Prevention of hypoxia-induced death 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) Inhibition of thromboxane A2, exerted by compound **96c**. The latter showed also anti-atherogenic properties.
- 3) Diuretic action exerted by the compound **97c**.
- 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 anti-inflammatory, anesthetic and anticonvulsant activity. For 1-lupinylbenzotriazoles (**98**, Fig. 59) the anti-inflammatory, diuretic, analgesic and anti-hypertensive activity was evaluated, obtaining good results in one or more areas [257]. Instead, compounds *N*-[2-(tert-amino)ethyl]- (**99**) and *N*-(lupinyl)-benzotriazol-1/2-yl acetamides (**100**), assessed as local anesthetics, showed an activity comparable or higher to lidocaine [258].

The benzotriazole ring was also combined with tetrazole systems in order to enhance the antinociceptive and anti-inflammatory properties of both nuclei [259]. Among the reported 1-[2-(1*H*-tetrazol-5-yl)ethyl]-1*H*-benzo[*d*][1,2,3]triazoles derivatives (**101a–c**), compounds (**101a**) and (**101b**) (Fig. 60) emerged for their antinociceptive properties, being able to triple and quadruple, respectively, the reaction time in the hot plate test. Instead, compounds (**102a**) and (**102b**) had anti-inflammatory activity superior to derivatives from the same series (Fig. 60). Finally, compounds (**101a–c**) were evaluated for their *in vitro* antibacterial, antifungal and anticonvulsant activity; obtained results showed moderate protection from seizures. Best results were reported for compound (**101c**) [260].

Among the new derivatives which can fit in the category of non-steroidal anti-inflammatory agents there were a few compounds obtained by the combination of benzotriazole and benzofuranic nucleus [261]. Later on, their antinociceptive and anti-inflammatory activities were evaluated. The 3-acetyl-4-(1-benzotriazolyl)-5-(2-benzofuryl)-1-(*p*-chlorophenyl)pyrazole (**103a**) stood out for its analgesic action, and SAR studies showed that the pyrazole nucleus was more effective than the 1,3,4-thiadiazole ring. The results obtained in several animal models suggested that these derivatives exerted centrally and peripherally mediated antinociceptive properties. Almost all 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 (**104a**). SAR analysis for 5-acetyl-1,3,4-thiadiazole derivatives demonstrates that such activity decreases with the modification on the phenyl side chain. Finally, studies on the mechanism of action showed that new compounds acted through a selective inhibition of COX-2 [262]. Anticonvulsant activity was checked for selected compounds. Similarly to reference drug valproic acid, derivatives (**104a,b**) were found to be active in subcutaneous metrazole (ScMet) test. However, only derivatives (**103b**) and (**104c**) were active in maximal electroshock seizure (MES) test similarly to the second reference compound phenytoin. None of them acted as the third reference compound carbamazepine, which was active both in MES and in ScMet tests [262]. Structures are depicted in Fig. 61.

#### 14. Benzotriazole as antiviral agent

Viruses are pathogens that can cause major diseases both in human and animals and determine life lost, economic losses and higher productivity costs. Bartorfd's classification part them in DNA and RNA viruses. Many biological targets against which antiviral compounds most likely would be successful have been identified. Crucial metabolic enzymes, such as polymerase, protease and helicase, represent an attractive target for drug discovery. Nucleosides and non-nucleosides inhibitors are widely investigated to implement the pharmacological arsenal and to obtain more potent and selective antiviral agents. Several benzotriazole derivatives

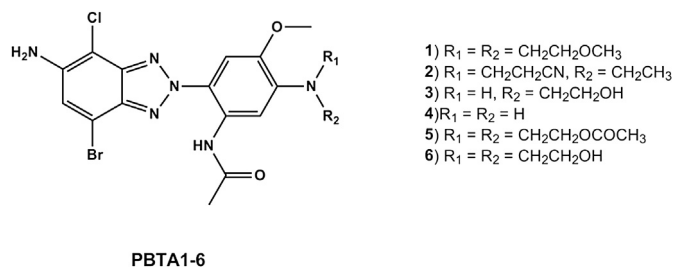


Fig. 73. Phenyl benzotriazoles derivatives recognized as mutagenic agents.

acted as antiviral agents, showing sometimes an interesting selectivity of action.

Synthesis and biological activity of benzotriazole analogous as NTPase/helicase inhibitors was extensively investigated [263]. As recently reported, NTPase/helicase is a promising target and ssRNA<sup>+</sup> (positive sense single-stranded RNA) enzymes were studied in detail [264]. ssRNA<sup>+</sup> viruses belonging to Flaviviridae and Picornaviridae families continue to pose threats to public health [265,266]. 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-( $\beta$ -D-ribofuranosyl) benzotriazole (**DRBt**) displayed an antiviral activity, showing  $\text{IC}_{50}$  values of 20 and 1.5  $\mu\text{M}$ , respectively [28]. (**DRBt**) and (**TBBt**) have been tested in four different HCV subgenomic replicon systems. Both of them resulted able to inhibit HCV replication ( $\text{EC}_{50}^{\text{DRBt}} = 10\text{--}53 \mu\text{M}$ ,  $\text{EC}_{50}^{\text{TBBt}} = 40\text{--}65 \mu\text{M}$ ) in a comparable way to the inhibition reported in the enzymatic assays, showing a property that has been detected only for a handful group of HCV inhibitors. Moreover, they were not cytotoxic at concentrations up to 100  $\mu\text{M}$  [267]. Furthermore, N-alkyl derivatives of (**TBBt**) showed good inhibitory activity against HCV, WNV, and JEV NTPase/helicases, and less cytotoxicity [268]. The benzotriazole moiety relevance was finally demonstrated by replacing it with a benzimidazole in the 5,6-dichloro and 5,6-dibromo-1-( $\beta$ -D-ribofuranosyl) benzimidazole (**DRB** and **DBRB**), which was much less potent as HCV helicases inhibitors (Fig. 62).

To explain this behavior, Bretner et al. synthesized and studied a new series of N-substituted 1H-benzimidazole and 1H-benzotriazole [263]. The starting compounds 1H-benzotriazole and 1H-benzimidazole showed a very low anti HCV-helicase activity on a DNA substrate ( $\text{IC}_{50}$  200  $\mu\text{M}$  and 500  $\mu\text{M}$ , respectively) and no activity ( $\text{IC}_{50} > 500 \mu\text{M}$ ) when measured either with an RNA substrate or against the enzymes of WNV, DENV, and JEV (Flaviviridae

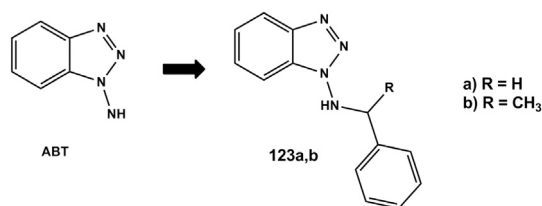


Fig. 75. Benzotriazole derivatives as isoform-selective suicide inhibitors of cytochrome P 450.

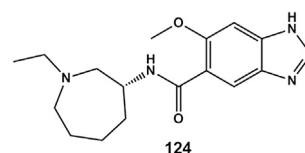


Fig. 76. Benzotriazole derivative endowed with strong antiemetic activity.

family). On the contrary, the whole brominated 1H-benzotriazole revealed to be 9–10 fold more effective as HCV helicase inhibitor when determined with an RNA or DNA substrate (more potency in case of JEV helicase,  $\text{IC}_{50}$  20  $\mu\text{M}$ ). Moreover, the brominated 1H-benzimidazole resulted to be less efficient than **TBBt** but more potent than the non-halogenated parent compounds against HCV helicase. To enhance hydrophobicity, N-alkylated derivatives (**105**) (substituted with methyl, ethyl and propyl moieties) were synthesized, as shown in Fig. 63.

The comparison of 1- or 2-alkyl benzotriazoles effectiveness on the HCV-helicase using the DNA substrate demonstrated that the 2-alkylated derivatives resulted significantly more potent as helicase inhibitors. In 1-alkylated benzotriazoles and benzimidazoles the aliphatic chain elongation determined an activity enhancement. However, the benzimidazole derivatives inhibitory activity was very low on DNA substrate (ranged between 250 and 500  $\mu\text{M}$ ), and inefficient on RNA substrate, as well as using other viral NTPase/helicases. This behavior suggested that these inhibitors do not act through a block on the enzymatic NTP binding sites. As previously suggested by Porter [269] the occupation of an allosteric nucleoside binding site should be considered.

Investigating the role of hydrophobic N-alkyl substituents, authors observed that replacement of the alkyl side-chain by a substituent endowed with higher hydrophilicity (such as in hydroxyethyl derivatives, **106**) or higher hydrophobicity (such as in chloroethyl derivatives, **107**) severely decreases the activity of **TBBt** derivatives. They demonstrated that a small hydrophobic alkyl

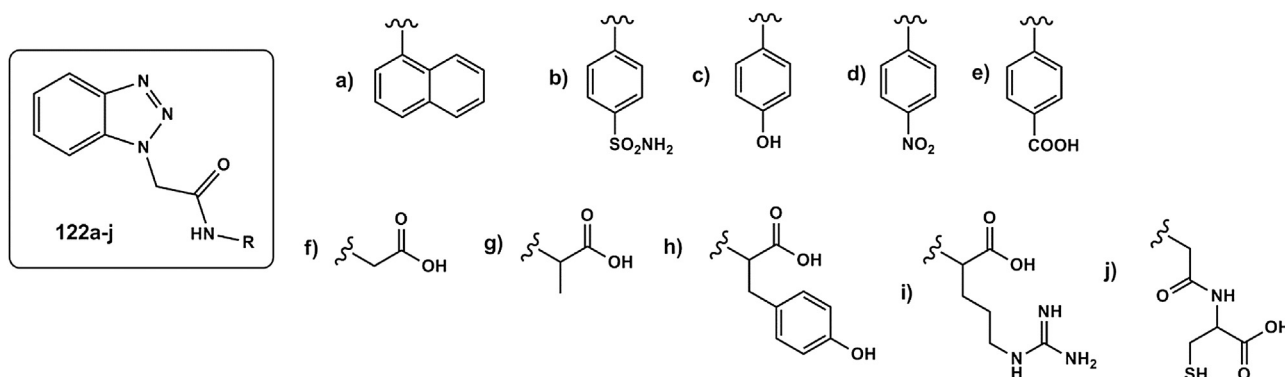


Fig. 74. Benzotriazole substituted with N-phenylacetamide endowed with high antioxidant activity.

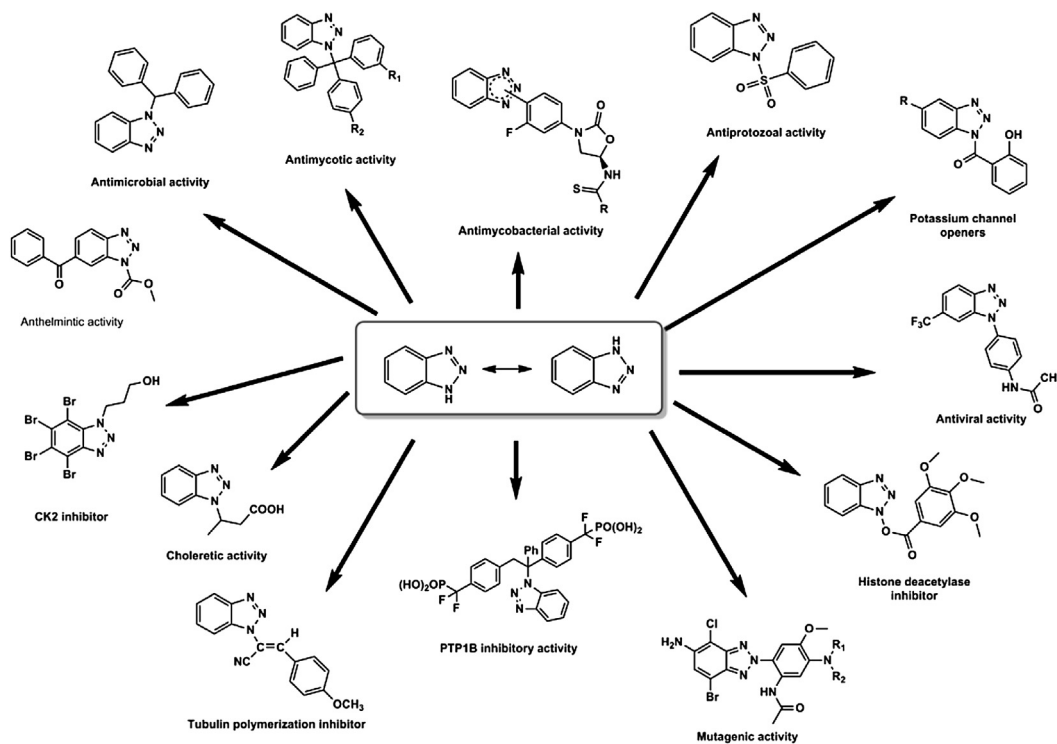


Fig. 77. Resuming scheme on benzotriazole derivatives possible biological activity.

moiety (methyl or ethyl) at TBBT position 2 could play a crucial role in the HCV NTPase/helicase inhibition. Finally, the introduction of a ribofuranosyl ring in both benzotriazole and tetrabromobenzotriazole improved the water solubility while led to an inhibitory activity decrease of the inhibitory activity against HCV and all the tested enzymes [28].

More NS3-targeting benzotriazole were investigated by Carta and coworkers. They evaluated the *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 (**108**) [270]. Enteroviruses CVB-2 and Sb-1 (Picornaviridae family) were the only viruses inhibited by mentioned compounds. Particularly, Picornaviruses represented an interesting target to explore, since actually no specific antiviral therapy is available for the treatment of Picornaviridae infections. Above all, two compounds emerged for their selectivity: *N*-(4-(5,6-dimethyl-2*H*-benzotriazol-2-yl)phenyl)acetamide (**109**), the most active against CVB-2 ( $EC_{50} = 10 \mu\text{M}$  and  $CC_{50} > 100 \mu\text{M}$ ), and *N*-(4-(6-(trifluoromethyl)-1*H*-benzotriazol-1-yl)phenyl)acetamide (**110**), the most active against Sb-1 ( $EC_{50} = 30 \mu\text{M}$  and  $CC_{50} = 90 \mu\text{M}$ ) (Fig. 64).

SAR analysis suggested that the potency and selectivity of compound (**109**) was probably due to the small electron donor methyl group at positions 5 and 6, a phenyl group at position 2 and a propanoyl-amide group in 4'. On the other side, the selectivity of compound (**110**) was correlated to a  $\text{CF}_3$  group in position 6, a phenyl in position 1 and an amide group in 4'. These 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 were able to rank binding affinities with a similar docking mode in the putative binding site (Fig. 65).

Using these molecules as lead compounds and making use of Diana's patent concerning *N,N'*-bis[4-(2-benzimidazolyl)phenyl]alkyldicarboxamides [271], Carta et al. prepared a series of *N,N'*-bis[4-(1*H*(2*H*)-benzotriazol-1(2)-yl)phenyl]alkyldicarboxamides

[272]. Evaluated in parallel cell-based assays for cytotoxicity and antiviral activity against representative of Picornaviridae and Flaviviridae, none of the (1*H*) derivatives resulted active against representative RNA viruses. On the contrary best activities were reported for (2*H*) analogues. Among them, bis-5,6-dimethyl-derivatives (**111a–c**) exhibited good activity against Enteroviruses ( $EC_{50} = 7–11 \mu\text{M}$  against CVB-2,  $EC_{50} = 19–52 \mu\text{M}$  against Sb-1). Finally, some bis-5,6-dichloro-benzotriazol-2-yl derivatives (**111d–f**) emerged for their interesting selective activity against CVB-2 ( $EC_{50} = 4–11 \mu\text{M}$ ), being totally inactive against all the other viruses screened (Fig. 66).

Finally, an extended binding mode study was performed on Sb-1 helicase, which model was previously obtained through homology techniques. Furthermore, in absence of CVB-2 helicase 3D model, the activity of (**111d–f**) 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) [273]. Spreading from southern China in late 2002, in 2003 SARS became epidemic and rapidly spread from its origin to more than 25 countries. It affected almost 8000 patients and cause the death of about 10% of those infected, reaching a high mortality rate [274].

Since no effective anticoronaviruses therapeutics are available, considering the lingering danger to human health represented by SARS-CoV as well as other Coronaviruses, the looking for helpful SARS-CoV antivirals is actually a focal point. The most important enzymatic target that the scientific community identified is represented by a protease, the main proteinase or  $\text{M}^{\text{pro}}$  (also called dimeric chymotrypsin-like protease or  $3\text{CLpro}^{\text{pro}}$ ) [275]. This enzyme cleavage two polyproteins, pp1a and pp1ab [276,277], to provide the functional proteins for viral propagation. Only few  $\text{M}^{\text{pro}}$  inhibitors have been reported, like aryl boronic acid [278], ketoglutamine analogues [279], phthalhydrazide ketones [280], an  $\alpha,\beta$ -epoxyketone [281], thiopurine analogues [282].

In 2006, Wu and colleagues reported a new class of stable benzotriazole esters, which appeared to act as 3CL<sup>pro</sup> irreversible inactivators, with inhibition constants in the nanomolar range [283]. All derivatives were obtained using the strategy of combinatorial reaction in microtiter plates followed by *in situ* screening [282–284]. Particularly, benzotriazole esters were prepared by condensation of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) with 90 various carboxylic acids. Only the derivatives which resulted stable at pH 5.0–8.0 over 24 h at room temperature were screened. All compounds were not cytotoxic (CC<sub>50</sub> on Vero E6 cells > 100 μM), and the 1*H*-indole-5-carboxylic acid benzotriazol-1-yl ester (**112**, Fig. 67) stood out, exhibiting a  $k_{inact}$  of 0.0011 s<sup>-1</sup> and a  $K_i$  of 7.5 nM.

An extensive mode of action study suggested that these benzotriazole esters could determine inhibition of 3CL<sup>pro</sup> *via* irreversible acylation of Cys145, as shown in Fig. 68. Particularly, Cys145 and His41 were essentials at the protease active site. They acted as the principal catalytic residues, in which the cysteine thiol operate as nucleophile in the proteolytic process [284]. However, even though the registered  $K_i$  values were among the lowest for any inhibitor of the SARS-CoV 3CL<sup>pro</sup> described so far, authors also reported that full inhibition of the enzyme could not be achieved.

Finally, additional benzotriazole esters proposed in the paper were developed to obtain more stable derivatives, in which ester oxygen is replaced by a carbon atom. Unfortunately they resulted weaker than the corresponding esters and they bind non-covalently 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 (**XP-27**) and 1-(4-dimethylaminobenzoyloxy)-benzotriazole (**XP-59**) [285]. These derivatives, which structures are depicted in Fig. 69, were able to inhibit the enzymatic activity at low micromolar concentrations (<5 μM and 0.1 μM for XP-27 and XP-59, respectively). Authors demonstrated that benzotriazole esters acted as suicide inhibitors by covalently binding to the proteinase.

Notably, from a library of inhibitors based on a halopyridinyl ester, compound (**113**) (Fig. 69) was able to completely inhibited Hepatitis A virus (HAV) 3C<sup>pro</sup> at nanomolar concentration [286].

So far, benzotriazole derivatives were 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 (Paramyxoviridae family). RSV is the leading cause of acute respiratory tract infections in all ages people but can also cause serious lower respiratory tract infections in infants younger than 6 months, in infants born before 35 weeks of gestation, in infants and in children with underlying lung disease or congenital heart disease [287], in 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 [96]. 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 collocated the paper written by Yu and coworkers. In 2003 the group reported 1-[(dialkylamino)alkyl]-2-[(benzotriazol-1/2-yl)methyl]benzimidazoles (**114**) as a new class of inhibitors able to protect HEP-2 human lung carcinoma cell line cells against RSV cytopathic effects [288]. Indeed, these compounds were protected by a Bristol-Myers Squibb Company patent [289],

but a part of them were firstly disclosed by Pagani [247] and Paglietti [290], who found them to possess analgesic and anti-arrhythmic activity. Lead compounds from this first series are depicted in Fig. 70 as new RSV-inhibitors. Particularly, 2-(2-((2*H*-benzo[*d*][1,2,3]triazol-1/2-yl)methyl)-1*H*-benzo[*d*]imidazol-1-yl)-*N,N*-diethylethanamines (**115–116**) stood out for their potent antiviral activity against both A and B RSV subtypes. A good cytotoxic profile was paired with EC<sub>50</sub> values an order of magnitude lower than that of ribavirin (EC<sub>50</sub> = 2.7, CC<sub>50</sub> = 34 μM, data not shown) [291].

SAR analysis were centered to the variation of the dialkylaminoalkyl side chain. A wide tolerance to structural variation was observed, and both polar and lipophilic groups at the terminal chain terminus preserved the RSV inhibition. However, the only requirement is a minimum of two atoms of separation between terminus and the heterocycle moiety. Authors also established that the topological relationship of benzotriazole in respect to the substituted benzoimidazole moiety is not critical since both isomers proprieties were equal [288,292].

Unfortunately for our review, the broad survey designed to delineate the pharmacophore associated with lead compounds guided Yu et al. to replace benzotriazole with a benzimidazol-2-one. This modification led to even more potent anti-RSV agents [293].

However, Tonelli et al. extended the analysis to 1-substituted-2-[(benzotriazol-1/2-yl)methyl]benzimidazoles, in accordance with the report from Bristol researchers. They evaluated the antiviral activity of related 1-substituted 2-[(benzotriazol-1/2-yl)methyl]benzimidazoles (**117**), 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. 71.

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 a few cases with EC<sub>50</sub> values below 50 nM [294]. SAR studies suggested that the presence of substituents at benzimidazole position 5 led to an increased cytotoxicity, especially if compound bear the (quinolizidinyl)alkyl nucleus. Toward antiviral activity, the most potent compounds carried a chlorine atom in position 5 paired with an  $R_1$  equal to (CH<sub>2</sub>)<sub>2</sub>N(CH<sub>3</sub>)<sub>2</sub> or (CH<sub>2</sub>)<sub>3</sub>N(CH<sub>3</sub>)<sub>2</sub>. On the contrary, the replacement of Cl with H, NO<sub>2</sub>, CF<sub>3</sub> or COCH<sub>3</sub> led to a potency reduction. Surprisingly, the introduction at  $R_1$  of bulkier group led to a progressive increase of potency, as seen when (CH<sub>2</sub>)<sub>2</sub>N(CH<sub>3</sub>)<sub>2</sub> was replaced with (CH<sub>2</sub>)<sub>2</sub>N(CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub> or lupinyl group, and homolupinyl derivatives were the most potent, regardless of whether  $R$  was H or Cl. Finally, N(2)-substituted benzotriazoles were generally less active than N(1)-substituted isomers.

## 15. Benzotriazole as PTP1B inhibitors

Diabetes mellitus type 2 (formerly noninsulin-dependent diabetes mellitus, NIDDM, or adult-onset diabetes) is a metabolic disorder of carbohydrate metabolism. It is characterized by high blood glucose levels due to defect in insulin secretion, insulin resistance or both [295]. The available anti-hyperglycemic agents actually are burdened with significant side effects, thus there is an urgent need to develop new therapeutic agents.

A promising biological target was identified in the protein tyrosine phosphatase 1B (PTP1B), which act as negative regulator in insulin signaling pathways. In years the protein structure was

elucidated and PTB1B inhibitors able to bind to the known active site 1/A were identified [296]. Puius and colleagues identified even an additional non catalytic aryl phosphate binding site (2/B) [297]. Consequently, new inhibitors able to bind across both site A and B were designed and synthesized.

With the aim to identify new inhibitors able to bind both sites A and B, a series of benzotriazole derivatives containing a difluoromethylphosphonates (DFMP) moiety was synthesized [298,299]. Compounds (**118**) and (**119**) (Fig. 72) emerged for their PTP1B inhibitory activity at nanomolar level ( $IC_{50} = 5$  nM and 15, respectively). Moreover, crystallographic analysis demonstrated that (**118**) acts as dual-inhibitors, exhibiting moderate selectivity for PTP1B over the closely related T-cell protein tyrosine phosphatase (TCPTP). Mutagenesis data confirmed these findings [300]. On this class of benzotriazole derivatives even 3D-QSAR and 3D-QSSR studies were carried out. Analysis results highlighted the relevance of the hydrogen bond formed by Arg 24 in PTP-1B and benzotriazole moiety for the class selectivity [301].

Novel tetrasubstituted benzotriazole-based PTP1B inhibitors (**120**, Fig. 72) containing a DFMP-substituted naphthyl template were prepared by Patel and colleagues. All compounds contained key structural components: a) benzotriazole ring, to obtain superior PTP1B selectivity; b) acetophenone, and c) benzyl, naphthyl, or quinolinyl ring systems, suitably substituted with difluoromethylsulfonamide (DFMS) to improve lipophilicity and oral efficacy. Finally, d) DFMP-substituted naphthyl or quinolinyl derivatives, to obtain compounds bearing two DFMP groups able to access both binding sites A and B, thereby improving potency and selectivity for PTP1B over TCPTP. Best results were obtained for compound (**121**) which showed excellent anti-hyperglycemic effects in animal models and improved oral bioavailability, along with excellent selectivity over T-Cell Protein Tyrosine Phosphatase (TCPTP) [302].

## 16. Mutagenic, antioxidant and antiemetic activity of benzotriazole

Phenyl benzotriazoles derivatives (**PBTA1-6**, Fig. 73) were identified as environmental pollutants endowed with mutagenic activity on *Salmonella* [303]. Considering that genotoxic compounds may play a role in the development of various diseases such as cancer, SAR analysis on PBTA, all showing comparable mutagenicity, highlighted the relevance for a primary amino group [304–308], along with the planarity of the 2-phenylbenzotriazole ring and halogen groups [309].

The potentially damaging effects of oxidative stress in cells is due to free radicals or reactive oxygen species (ROS) that are generated as result of cellular metabolism, i.e. during infections. Antioxidants are agents whose action is to limit oxidative damages [310]. Benzotriazole derivatives of N-Phenylacetamide (**122a–e**) and acetylcarbamic acid (**122f–j**) were synthesized and their antioxidant activity was determined following radical activity of nitric oxide in biological systems using Griess reaction. In the presence of nitric oxide, synthesized compounds (**122a–j**, Fig. 74) were able to reduce the production of nitrite ion, a strong oxidizing agent, due to the presence in their chemical structure of carbonyl and amine functional groups, able to interact with nitric oxide.

Benzotriazoles substituted with N-phenylacetamide showed higher antioxidant activity than analogues substituted with acetylcarbamic acid. Above all, compounds (**122b**), (**122c**) and (**122e**) showed remarkable scavenging activity when compared to ascorbic acid [311].

N-arylkylated derivatives of 1-aminobenzotriazole (ABT) (Fig. 75) were synthesized as isoform-selective suicide inhibitors of cytochrome P 450. N-benzyl (**123a**) and N- $\alpha$ -methylbenzyl (**123b**)

ABT derivatives showed the greatest potency, and the latter was the most efficient isoform-selective suicide inhibitor [312]. The order of inactivation for isozyme monooxygenase for lung P450 1A1, 2B4 and 4B1 orthologues was 2B4 > 1A1 >>> 4B1 [313]. N-arylkylated derivatives appear to inactivate the P450 2B isoforms by binding to the apoprotein [314,315].

Finally, antiemetic and gastroprokinetic activities were evaluated for a series of 6-methoxy-1H-benzotriazole-5-carboxamide derivatives with a medium perhydroazacycle ring in the amine moiety. These molecules were designed with the aim to obtain a peripheral dopamine D2 receptor antagonist. Particularly, derivative (**124**, Fig. 76) exhibited a strong antiemetic activity ( $ED_{50} = 0.08$  mg/kg, p. os) without 5-HT3 receptor antagonistic activity. On this class of compounds an extended SAR analysis and a pharmacological profile evaluation were performed [316,317].

## 17. Conclusion

The discovery of novel drugs in many fields, i.e. antibacterial, has been stalled for many years. There is an urgent need for new pharmaceuticals which have a broader spectrum of activity or act through novel mechanisms of action, i.e. to overcome the increasing incidence of microbial resistance observed for currently used drugs. Benzotriazoles are regarded as a promising class of bioactive heterocyclic compounds that exhibit a range of biological activities. Therefore, this nucleus appears a very interesting scaffold in the drug discovery and development processes. As proved in this paper, benzotriazole is useful to wide develop SAR analysis on different classes of pharmacological agents. More than 13 possible biological activities for benzotriazole derivatives were here reported (Fig. 77).

Isosteric modifications done through the introduction of benzotriazole moieties were sometimes successful, lowering activity at nanomolar concentration. More than one hundred molecules are here presented as virtually hit and lead compounds for further drug development. However, despite the active, exhaustive and target based research on development of many compounds as antibacterial, antimycotic, antimycobacterial, potassium channels activators, etc. no molecule has made its way to the market and clinic. It can be probably due to lack of molecular targets knowledge through which most of those compounds exert their biological actions, but it can be also due to lack of a comprehensive compilation of various research reports in each activity capable of giving an insight into the SAR of the compounds. The present review, covering more than 310 references, is expected to provide a low-height flying bird's eye view of the benzotriazole derived compounds to a drug designer and medicinal chemist for a comprehensive and target oriented information for development of clinically viable molecules.

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