

Review

The Structure and Activity of Double-Nitroimidazoles. A Mini-Review

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Received: 10 June 2018; Accepted: 16 July 2018; Published: 25 July 2018



Abstract: Many interesting applications have been found for nitroimidazoles as therapeutic agents. Among others, some of these compounds can radiosensitize hypoxic tumor cells. The introduction of a second nitroimidazole ring to the molecule can improve the level of its pharmacological effect. The aim of this article is to overview the literature concerning active compounds that contain two nitroimidazole moieties in their structures.

Keywords: nitroimidazoles; double-nitroimidazoles; bis-nitroimidazoles; tumor hypoxia; radiosensitizers

1. Introduction

The antibiotic azomycin was the first active nitroimidazole to be discovered [1]. Its discovery provided the major impulse for the systematic search for medicines showing activity against anaerobic protozoa. This research led to the acquisition of the 5-nitroimidazole derivative—metronidazole, which is most widely used in the treatment of anaerobic protozoan parasitic infections caused by *Trichomonas vaginalis*, *Giardia duodenalis*, and *Entamoeba histolytica* [2,3].

A common feature of tumors is an imbalance between oxygen (O₂) supply and consumption, called hypoxia, the severity of which varies among tumor types [4,5]. In tumor tissue, which becomes larger and proliferates, oxygen demand is exceeded by oxygen supply, and the distance between cells and the existing vasculature rises, limiting oxygen distribution. Initial oxygenation of the tissue as well as the dimension and point in tumor development are connected with oxygen levels in the tumor. Main determinants of tumor hypoxia are atypical structure and function of the micro-blood vessels which supply the tumor, increased diffusion distances between the nutritional microvessels and the tumor cells, and reduced oxygen transport capacity of the blood because of the existence of disease- or treatment-related anemia. Tumor hypoxia might diminish the effectiveness of radiotherapy, some O₂-dependent cytotoxic agents, and photodynamic therapy.

The inability of surrounding vasculature to keep pace with the tumor growth is a main factor leading to the development of hypoxic regions in tumors [6]. Radiosensitizers have been shown to enhance tumor damage by selectively increasing the radiosensitivity of hypoxic cells. Many nitroimidazole derivatives have been found to sensitize hypoxic cells to the lethal effect of ionizing radiation when present at the time of irradiation [7]. When a nitroimidazole derivative passes into a viable cell by diffusion, the compound undergoes a set of chemical alterations: initially, the molecule attains a single electron in the cytoplasm by an enzymatic process to create a potentially reactive species. Subsequently, the molecule is immediately reoxidized in the presence of adequate intracellular O₂ levels [8]. This process is repeated until the whole compound diffuses out of the cell. In the absence of an adequate supply of O₂, in hypoxic tissue, they undergo further reduction to more reactive products that bind to cell components because the decreased oxygen concentration is not able to effectively compete to reoxidize the molecule. An enzyme-mediated, single-electron

reduction of the NO₂ group initiates the formation of these products. The nitro group is reduced to a free radical, which exists in form of an anion at neutral pH. The reduction pathway can proceed in successive steps past the NHOH derivative to terminate at the relatively inactive amine derivative. The reoxidation does not occur with low oxygen concentration. The reactive molecules subsequently go through additional reduction pathways and remain in the cell. Reduction takes place in all tissues with viable enzymatic processes, but retention only exists in those tissues with reduced oxygen levels.

In addition to their radiosensitizing activity, nitroimidazole derivatives are selectively toxic to hypoxic cells because of their reductive activation via oxygen-inhibitable metabolic pathways [9]. One of the most investigated types of compounds are 2-nitroimidazole derivatives. They undergo enzymic, oxygen-inhibitable reduction of NO₂ group, resulting in preferential metabolism in hypoxic cells to reactive metabolites [10]. The introduction of a second nitroimidazole moiety into the molecule results in obtaining the double- or bis-bioreductive agent, in which the inhibitable reduction of two centers would be required for full cytotoxicity. The aim of this review is to present the current state of knowledge about molecules that contain two nitroimidazole rings in the structure. There are two types of such compounds: double-nitroimidazoles with unsymmetrical structures and bis-nitroimidazoles, with the same two nitroimidazole moieties which form symmetrical molecules or possess two symmetrical parts.

All the available biological test results for described compounds are summarized in Table 1.

Table 1. The activities of presented double- and bis-nitroimidazoles.

Compound No.	Reported Activity	Reference	
2	Cytotoxicity: hypoxic CT ₁₀ (mM-h) 0.37–12 Radiosensitization: uptake C _i /C _e 0.33 C _{1,6} (mM) 0.63 C _{1,6(i)} (mM) 0.21	[11]	
4	In vitro aerobic cytotoxicity and hypoxia—selectivity growth inhibition assay: IC ₅₀ (mM) 2.00 ± 0.92	[10]	
5			IC ₅₀ (mM) 1.00 ± 0.17
6			IC ₅₀ (mM) 1.18 ± 0.37
7			IC ₅₀ (mM) 0.81 ± 0.23
8	Inhibition of polyamine uptake; inhibition constants: K _i (μM) 5.0	[12]	
9			K _i (μM) 0.6
12	Fluorescent data; FL (Φ) (λ _{max} /nm) 455.5	[13,14]	
13			(λ _{max} /nm) 404.5
16	Maximum fluorescence concentration (μM), measured postinjection of 25 μM were: 0.064 (1 min); 0.131 (15 min); 0.111 (30 min); 0.105 (60 min); 0.072 (120 min); 0.01 (180 min); 0.044 (300 min); 0.044 (420 min)	[15]	
17	Fluorescence quantum yield: Φ = 0.467	[16]	
18	SPECT/CT imaging confirmed that the tumors could be visualized clearly with comp. 18 at 2 h (conc. 22% ID/g)	[17]	

in which two molecules of well-known metronidazole are linked, has been presented in detail in Pawełczyk et al. [21] as a synthesis of “molecular consortia”.

In 1994, New Zealand scientists reported a procedure for the synthesis of double (nitroimidazolyl)alkanecarboxamides (Figure 2) [11]. These compounds were prepared by a direct coupling of side chain acid and amine components, with the use of diethyl phosphorocyanidate at room temperature. Further chemical transformations included obtaining the key intermediate, named 2-(2-methyl-5-nitro-1*H*-imidazolyl) ethylamine, by a set of chemical modifications, e.g., alkylation of imidazole with bromophthalimide derivative, which replaced the bromophthalimide moiety with hydrazine monohydrate to give imidazoleethylamines. The carboxamide-linked double-nitroimidazoles were obtained via a reaction of these amine derivatives with an acid component.

Linkage of the two nitroaromatic moieties was effected at 1-position through *N*-alkyl chains. Such linkers ensure electronic isolation, avoiding significant disorder of NO₂ reduction potentials. All the substances showed aerobic toxicities corresponding to those of mononitro derivatives. Concurrently, bis-nitroimidazoles act as radiosensitizers of hypoxic cells *in vitro*, but they are only slightly more active than the known mononitro derivatives. One of the main limitations in biological evaluation of these carboxamide-linker compounds was their poor solubility in water. The more soluble substance (2) was active as a radiosensitizer of murine fibrosarcoma KHT tumor cells when it was administered in multiple doses (Figure 2).

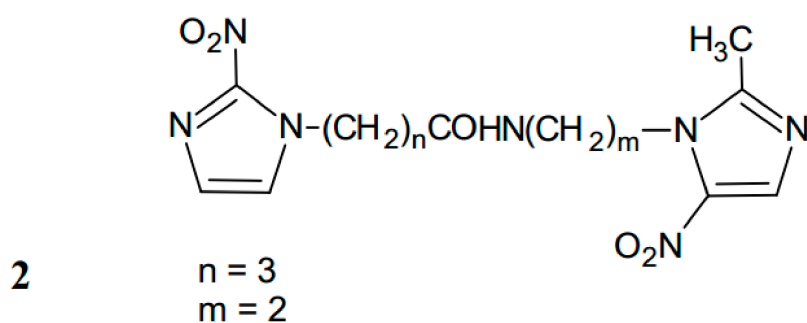


Figure 2. The structure of carboxamide-linker double-nitroimidazole.

The potency of 2 as a hypoxic cell radiosensitizer *in vitro* is no higher than that of mononitroimidazoles [9]. The kinetics of potential drug activation is consistent with the formation of a bifunctional cytotoxic species that does not act as a DNA cross-linking agent [10]. This substance was characterized by significantly higher (up to 200-fold) hypoxic selectivity than misonidazole in AA8 cells and is potent against KHT and MDAH-MCa-4 tumors in *in vivo* tests.

Further investigation was directed at increasing aqueous solubility and obtaining higher activation under hypoxic conditions. The study was extended by the synthesis and biological evaluation of double-nitroimidazoles derivatives with an isopropyl linker group, a series with glucopyranosyl linkers, which analogues to oxamide linkers, and a succinamide chain instead of a carboxamide linker [10]. Among these derivatives, only the aminoalkyl provided substances with sufficient solubility in water so that they could be evaluated fully in cell culture (Figure 3). The amino-linked 2-nitro/5-nitro analogs (3–5) were characterized by aerobic cytotoxicity two- to seven-fold higher than that of the carboxamide-linked analogue of compound 2. The compounds with symmetrical amine linkers in 6, 7 were from six to nine times more active than 2.

Other authors have described a synthetic method for obtaining novel polyamine-nitroimidazole conjugates [12]. It has been suggested that polyamines can be used as vectors for the cellular delivery of different DNA targeted substances. The introduction of nitroimidazole moiety to polyamines may deliver the drug to target tumor cells by the high specific activity. Moreover, polyamine can also increase the concentration of the drug near DNA, the proposed site of action of nitroimidazoles [12]. On the basis of such assumptions, Siddiqui et al. [12] obtained a series of

N^1,N^7 - and N^1,N^8 -bis-derivatised spermidine-nitroimidazole compounds (Figure 4), which were tested for their ability to be used as starting compounds for the polyamine uptake system in A549 lung carcinoma cells by measuring inhibition of [^{14}C]spermidine uptake. It was found that tested conjugates were promising in the targeting of tumor cells.

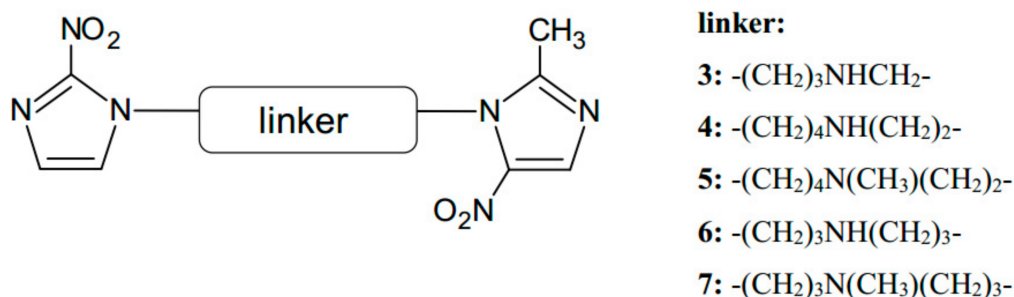


Figure 3. The structures of the most active aminoalkyl-linked analogs (3–7).

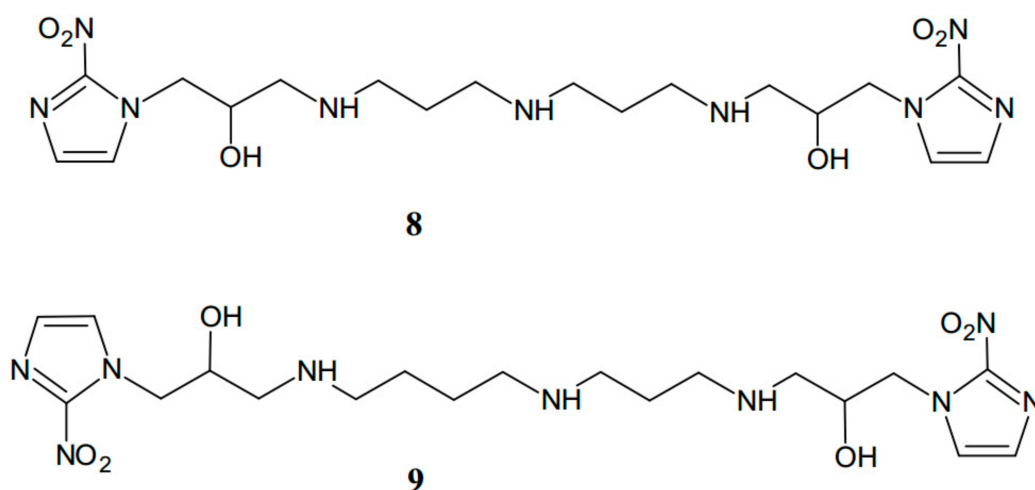


Figure 4. Structures of N^1,N^7 -bis-derivatised norspermidine-nitroimidazole conjugate (8) and N^1,N^8 -bis-derivatised spermidine-nitroimidazole conjugate (9).

In 2005, a method for obtaining bis(hydroxamamide)-based tetradentate ligands for $^{99\text{m}}\text{Tc}$ -radiopharmaceutical was described by Xu et al. [22]. They [22] proposed the synthesis of two new compounds: N,N' -ethylene bis (1-(4-nitroimidazole-1-yl)-propanhydroxyiminoamide) (10) and N,N' -propylene bis (1-(4-nitroimidazole-1-yl)-propanhydroxyiminoamide) (11) (Figure 5).

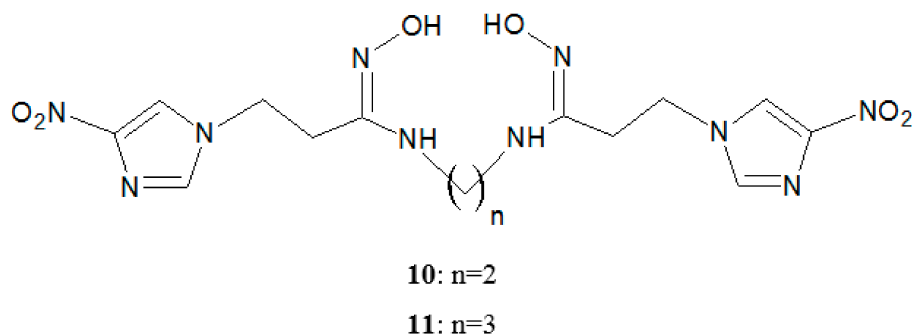


Figure 5. Structure of tetradentate ligands (10, 11).

These bis-nitroimidazoles were labeled with ^{98m}Tc forming appropriate complexes that are electrically neutral under physiological conditions. The results suggested that the obtained products may be useful as chelating molecules for the preparation of radiopharmaceuticals.

Qian and colleagues [13,14] have demonstrated that bis-(2-nitroimidazole)-naphthalimide derivatives (**12**, **13**) can act as fluorescent markers for hypoxia (Figure 6).

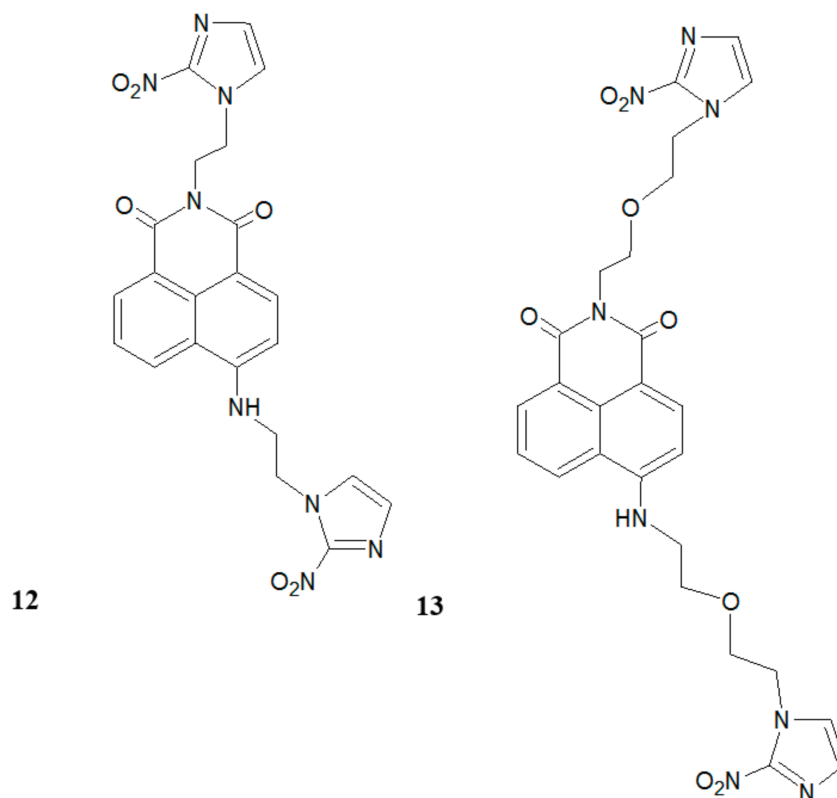


Figure 6. The structures of two bis-(2-nitroimidazole)-naphthalimides (**12**, **13**).

In biological tests, these compounds showed high fluorescence differences between hypoxic and normoxic cells. In particular, compound **13** was very active under hypoxic conditions.

Interestingly, some authors developed the methods of obtaining bis-/double- nitroimidazoles connected with metal atoms [17,23]. For example, Technetium-99m is used because of its properties which have ideal characteristics for medical scanning. It has a very short half-life, which is long enough to examine physiological processes, yet short enough to minimise the radiation dose to the patient. Also, it emits low energy γ radiation that reduces damage to tissues, but it can still be detected in a patient's body by a γ -ray sensitive camera. Moreover, Technetium-99m is quickly eliminated from the organism.

Giglio and colleagues developed the novel active ^{99m}Tc -radiopharmaceuticals for imaging hypoxia [23]. These products were obtained by the formation of Tc-nitrido-complexes with two new dithiocarbamate derivatives of metronidazole (Figure 7). It was found that only compound **14** showed adequate chemical stability together with low protein binding associated with fast blood clearance and moderate lipophilicity.

Another interesting approach to the synthesis and evaluation of bis-nitroimidazoles has been presented by Xu et al. [15], in which the authors described the method for obtaining a more expanded structure of novel nitroimidazole indocyanine dye conjugate (2-nitroimidazole-ICG) (**16**) (Figure 8) using a piperazine linker for tumor-targeted hypoxia fluorescence tomography [15].

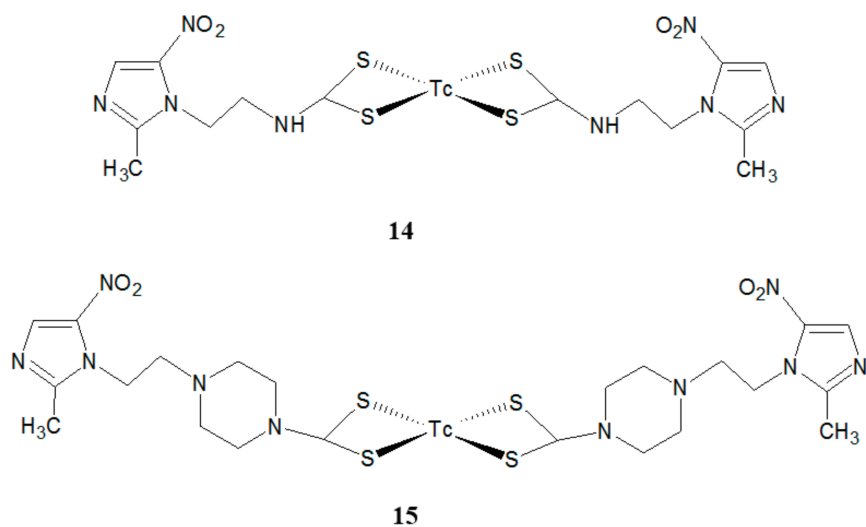


Figure 7. The structures of two new ^{99m}Tc complexes (**14**, **15**).

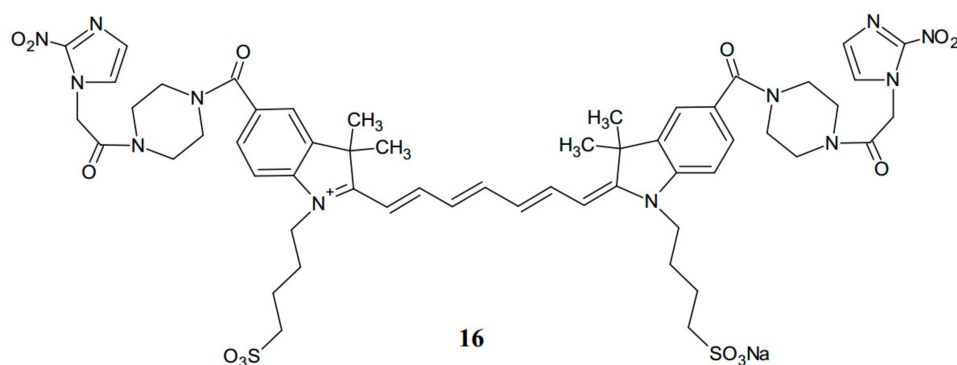


Figure 8. The structure of 2-nitroimidazole-ICG conjugate (**16**).

The activity of the new compound (**16**) was compared with that of the earlier known analogue of ethanolamine-2-nitroimidazole-ICG. Mouse tumors, located at imaging depths of 1.5 and 2.0 cm in a turbid medium, were imaged at various time points after intravenous injection of the dyes. As a result, the measured maximum fluorescence concentration of the tumors injected with compound **16** was two-fold higher than that after injection with ethanolamine-2-nitroimidazole-ICG within 3 h. Piperazine-2-nitroimidazole-ICG (**16**) is a novel second-generation tumor hypoxia-targeted conjugate. The results of biological evaluation suggest that the molecules which are more rigid and planar are characterized by higher fluorescence yield, and they might release less absorbed energy [16]. The same research group has reported a new substance after further chemical modifications with an improved level of fluorescence quantum yield. This third-generation conjugate (**17**) has two carbon atoms less in the polyene linker, and it is prepared by coupling reaction of the new dye with 2-nitroimidazole using a piperazine linker (Figure 9).

The fluorescence quantum yield is about five times higher than that measured for the second-generation hypoxia dye. Experiments *in vivo* were also promising. A new compound (**17**) showed more than two times the fluorescence intensity in the tumor as compared with a second-generation conjugate (**16**).

Other authors reported a method of obtaining the novel imaging agent, ^{99m}Tc ethylenedicysteinebis-misonidazole (^{99m}Tc -EC-MISO) (**18**) (Figure 10), for diagnosing tumor hypoxia [17]. The results of biological evaluation conducted on subcutaneous glioma-bearing mice were very promising. Single-photon emission computed tomography (SPECT/CT) imaging confirmed that the tumors can

be visualized clearly with ^{99m}Tc -EC-MISO at 2 h. Introduction of a second 2-nitroimidazole moiety caused an apparent hypoxic accumulation of ^{99m}Tc -EC-MISO imaging agent in the tumor.

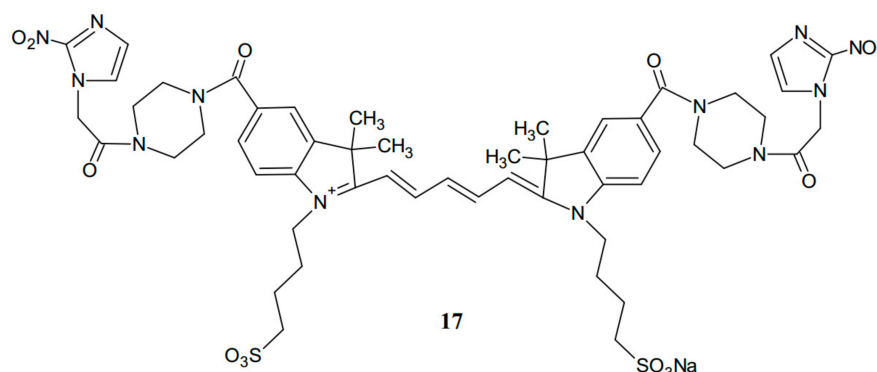


Figure 9. The structure of the third-generation conjugate (17).

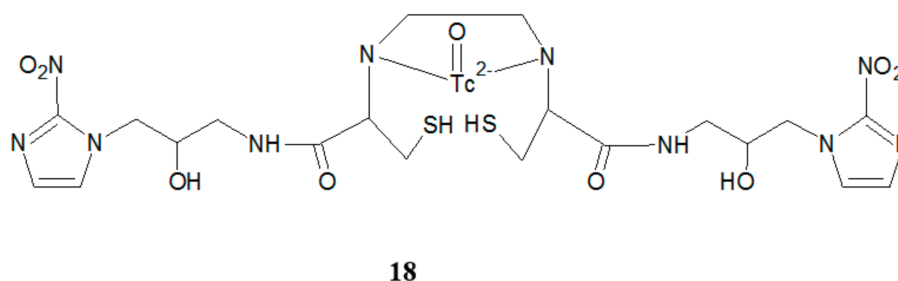


Figure 10. The structure of ^{99m}Tc -EC-MISO.

Girhepunje and colleagues have presented a set of new 5-nitroimidazole derivatives with antibacterial and antifungal activities [18]. One of these compounds, 1,4-bis{(2-methyl-5-nitro-1H-imidazole)-2-ethyl propanoate}piperazine (19), was particularly interesting (Figure 11).

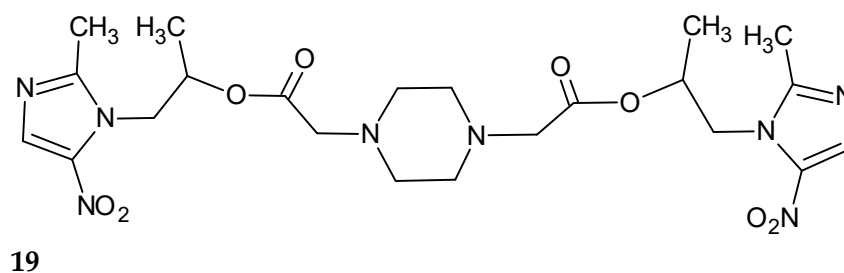


Figure 11. The structure of the compound (19).

In biological studies, it was found to show significant antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, higher than those evaluated for other tested compounds. Moreover, it had antifungal properties, especially against *Candida albicans* and *Aspergillus niger*.

Nitroimidazole derivatives are not only used as drugs in medicine but they also have other applications. The presence of more than one nitro group makes these compounds high-energy materials. Among them is 4,4',5,5'-tetranitro-2,2'-biimidazole (TNBI) (20), which can be used in explosive compositions because it has high detonation parameters [19]. This substance is very hygroscopic, and it can be used only in salt form (Figure 12).

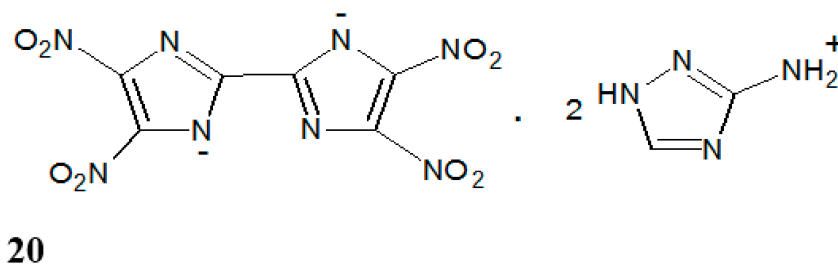


Figure 12. The structure of 3-amino-1,2,4-triazolium 4,4',5,5'-tetranitro-2,2'-biimidazolate (20).

As displaced Table 1 suggests, the main area of application of bis-/double- nitroimidazoles is their use in anticancer therapy. Concurrently, they have other interesting applications, e.g., antibacterial, antifungal, and nonmedical, such as explosives. Moreover, some of these compounds are used in the form of complexes with metals, especially with Technetium-99m. Such substances serve as radiopharmaceuticals.

3. Conclusions

Drugs containing a nitro group are widely used in the treatment of many illnesses and conditions e.g., hypertension, heart failure, coronary artery disease, and in the prevention of stroke [24].

Nitroimidazoles are categorized mainly as bioreductive prodrugs, characterized by both radiosensitizing and cytotoxic activities [25].

The NO₂ nitrogen atom in the molecule, which has an electron affinity, is responsible for radiosensitizing activities. The radiation-caused ionization and the NO₂ group undergoes reduction processes, in turn delivering oxidative damage to DNA, an oxygen-mimetic effect. Misonidazole, metronidazole, and etanidazole were placed in the group of the first nitroimidazole derivatives tested in clinical trials. Introduction of a second nitroimidazole moiety into the molecule results in the acquisition of the double/bis-nitroimidazole bioreductive agent, in which full cytotoxicity could be achieved by the inhibitable reduction of two centers. New compounds with two nitroimidazole moieties often have a more complicated structure, that is connected with the introduction to the molecule by means of biologically advantageous side chains or/and metal elements. Their desirable activities are significantly higher than the potencies of the simplest derivatives of double/bis-nitroimidazole.

Conflicts of Interest: The authors declare no conflict of interest.

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