Research article

Synthesis, characterization, and *in vitro* evaluation of gamma radiation-induced PEGylated isoniazid

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ABSTRACT

Background: The search for innovative anti-tubercular agents has received increasing attention in tuberculosis chemotherapy because *Mycobacterium tuberculosis* infection has steadily increased over the years. This underlines the necessity for new methods of preparation for polymer-drug adducts to treat this important infectious disease. The use of poly(ethylene glycol)(PEG) is an alternative producing anti-tubercular derivatives. However, it is not yet known whether PEGylated isonicotinylhydrazide conjugates obtained by direct links with PEG are useful for therapeutic applications.
Results: Here, we synthesized a PEGylated isoniazid (PEG-g-INH or PEG–INH) by gamma radiation-induced polymerization, for the first time. The new prodrugs were characterized using Raman and UV/Vis spectrometry. The mechanism of PEGylated INH synthesis was proposed. The *in vitro* evaluation of a PEGylated isonicotinylhydrazide macromolecular prodrug was also carried out. The results indicated that PEG–INH inhibited the bacterial growth above 95% as compared with INH, which showed a lower value (80%) at a concentration of 0.25 μM. Similar trends are observed for 0.1, 1, and 5 μM.
Conclusions: In summary, the research suggests that it is possible to covalently attach the PEG onto INH by the proposed method and to obtain a slow-acting isoniazid derivative with little toxicity *in vitro* and higher anti-mycobacterial potency than the neat drug.
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1. Introduction

Tuberculosis (TB) is a pandemic disease caused by the bacillus *Mycobacterium tuberculosis* (MTB) [1]. This airborne disease is in the top ten causes of death according to the World Health Organization (WHO) [2]. Despite millions of people being diagnosed and treated yearly for TB, there are still obstacles to overcome regarding treatment and resistance [3]. In 2018, the WHO published a report showing several drugs that are currently going through Phase I, II, or III trials [4]. However, the contemporaneous trends are highly arduous due to the long-time of chemotherapy necessary to eradicate this communicable infectious disease [5].

The emergence of multidrug resistance (MDR) strains has also shown that there is a need to continue working on tuberculosis drug-development [3]. The term extensively drug-resistant (XDR) tuberculosis demonstrates that this disease has no boundaries in the increasing resistance to new pharmaceuticals [6].

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Among the tuberculostatic agents, the antibiotic isoniazid, also known as isonicotinylhydrazide (INH) is considered the core or first-line drug for the treatment (chemotherapy) of active TB disease [7]. The INH activity against TB is remarkable [8]; it has to be noted, however, that isoniazid can cause liver injury (hepatotoxicity) [9], and exhibit adverse effects such as peripheral neuropathy [10]. Isoniazid must be administrated for long periods along with other drugs, for instance, pyrazinamide, rifampicin, and either ethambutol or streptomycin [11]. It is, therefore, necessary to chemically modify or combine the INH with other polymeric substrates to reduce the toxicity and/or increase drug activity, without further mandatory association with other tuberculosis medications [12].

To face this challenge, multiple derivatives of isoniazid have emerged in recent years, to mention a few examples, isoniazid by-products possessing 4-thiazolidinone and 2-azetidinone moieties [13], isonicotinoyl hydrazones [8], isonicotinoyl hydrazides [14], and N-alkyl-5-(pyridin-4-yl)-1,3,4-oxadiazole-2-amines [15]. On the other hand, the PEGylation of pharmaceuticals is an attractive alternative to produce prodrugs [16,17]. It is a technique that involves the grafting of poly(ethylene glycol) (PEG) onto target molecules and increases their efficiency and stability. Early studies were focused on the use of PEG for the fabrication of flexible poly(lactic acid)/poly(ethylene glycol)-functionalized polyhedral oligomeric silsesquioxane (PLA/PEG-POSS) nanocomposites [18], polyvinyl alcohol/poly(ethylene glycol) (PVA/PEG) hydrogels [19], and (acrylic acid/ PEG)-zinc oxide mucoadhesive using gamma-ray irradiation [20]. It would appear, therefore, that radiation-induced PEGylation could be an alternative to design new drugs mitigating TB. So far, there is a tendency to PEGylate macromolecules such as proteins to generate so-called PEG-protein conjugates [21], but the PEGylation of non-proteic molecules such as small pharmaceuticals remains under development [17].

Previously, several works have been carried out that involves the use of PEG in the preparation of INH derivatives, such as the copolymer PEG-poly(aspartic acid) prodrug with isoniazid [22], the conjugate of INH with methoxypoly(ethylene glycol)-b-poly(L-lysine) [23], the complex Cu–PEG–sucrose–INH [24], and various anti-tuberculosis macromolecular prodrugs based on INH conjugates with PEG derivatives [25]. The use of chelating agents to formulate PEG–bis(INH) conjugates [26] for infection diagnosis, and therapy [27] has also been reported. In this scenario, gamma-radiation-induced polymerization is a technique — based on the generation of free radicals — that allows the direct production *via* PEGylation of small molecules with no need for catalysts or initiators, which results in biocompatible polymer carriers of high purity [28]. In this work, we report the synthesis of PEGylated isoniazid by the simultaneous irradiation method. Gamma-radiation-induced PEGylated INH (PEG-g-INH or PEG–INH), the first of its kind to our knowledge, was structurally characterized by Raman spectroscopy and ultraviolet–visible spectrophotometry (UV–vis). It is also shown a proposal for the radiation-induced PEGylation mechanism of INH. Further *in vitro* evaluations of anti-mycobacterial activity and cell viability of the PEGylated derivatives were performed.

2. Materials and methods

Isonicotinylhydrazide (Synonym: 4-Pyridinecarboxylic acid hydrazide, Isonicotinic acid hydrazide, Isonicotinic hydrazide, INH) was purchased from Merck (MDL # MFCD00006426, St Louis, MO, USA) analytical standard, ≥99% (TLC) (Supelco). Double-distilled water was employed to prepare solutions of 12.5 mg/ml with PEG of 400, 1000, 3500, and 4000 Da, namely G1, G2, G3, and G4, respectively. All PEG was acquired from Sigma-Aldrich. Pyrex glass tubes, which contained roughly 1.25 g of INH and 2 ml of the previously mentioned PEG solutions (G1-G4) were subjected to a ^{60}Co-γ-radiation in the air (Gamma Beam 651 PT, Nordion International) source. The simultaneous irradiation method was utilized. It involves the direct exposure of INH and PEG solution to the high-energy of radiation, at a dose rate of roughly 1.5 kGy/h and doses of 5, 10, 15, 20 and 25 kGy, hereafter

named: D1, D2, D3, D4, and D5 accordingly. The dose rate was determined with a Fricke dosimeter. The PEG/INH solutions were degassed by 20 min of freezing in liquid nitrogen and the subsequent 3 h of thawing until three freeze–thaw cycles were completed. The test samples were labeled as M2G1D1, M2G1D2, M2G1D3, M2G1D4, M2G1D5, M2G2D5, M2G3D5, and M2G4D5, where M2 is assigned to INH (to simplify the names in the figures), while G and D represent variations in the molecular weight of PEG and the doses, respectively. For instance, M2G2D1 stands for PEGylated isoniazid with PEG of 1000 Da at 5 kGy. In addition, increasing concentrations of the sample M2G1D5 were set up to evaluate possible changes in the absorbance or wavelength in spectrophotometry. The samples stated to be 14 mg/ml (P1), 28 mg/ml (P2), 44 mg/ml (P3), 59 mg/ml (P4), 63 mg/ml (P5), 79 mg/ml (P6), 83 mg/ml (P7), and 100 mg/ml (P8) of PEGylated INH were labeled as M2G1D5P1, M2G1D5P2, M2G1D5P3, M2G1D5P4, M2G1D5P5, M2G1D5P6, M2G1D5P7 and M2G1D5P8 respectively. The samples were prepared in triplicate to ensure the accuracy of the experiments. Table 1 explains what the labels mean.

The Raman spectroscopy of INH, PEG, and PEGylated isoniazid was accomplished at ambient temperature in a micro-Raman spectrometer (Bruker Senterra, model 910, MA, USA) equipped with a 785-nm laser light source. A drop of the sample was placed on a flat and clean brass plate. This procedure is intended to avoid any interference enclosed by the support and the polymers solution signals.

The UV/Vis (150–650 nm) spectra of isoniazid (M2) and all PEGylated INH samples was measured using a microplate spectrophotometer reader (Multiskan GO, Thermo Fisher Scientific Inc., Waltham, MA, USA). Multimode wavelength scan at room temperature and with linear shaking was used.

Mycobacterium tuberculosis H37Ra was obtained from the American Type Culture Collection (ATCC 25177, Rockville, MD, USA). The bacterium was grown at 37°C in 7H9 broth (Difco, Detroit, MI, USA) supplemented with ADC (Beckton Dickinson, BD, San Jose, CA, USA) 0.2% (v/v) glycerol and 0.05% (v/v) Tween-80 or on solid Middlebrook 7H10 medium (Difco) supplemented with 0.5% (v/v) glycerol and OADC (BD) an 0.05% Tween-80 (Sigma-Aldrich, St. Louis, MO, USA).

The anti-mycobacterial activity was assayed in 96-well microplates. In doing so, the PEGylated and neat INH were added to wells at final concentrations ranging from 0.1 to 5 μg/ml. Then, each well was inoculated with a bacterial suspension containing 10^5 colony-forming units (CFUs)/ml for a total volume of 200 μl. The plates were incubated for 7 d at 37°C. Subsequently, the CFUs were counted as previously described [29], and the minimum inhibitory concentration (MIC) was determined.

Table 1
Labelling of the samples used for radiation-induced PEGylated isoniazid.

Samples	Molecular weight of PEG (Da)	Dose (kGy)	
M2G1D1	400	5	
M2G1D2	400	10	
M2G1D3	400	15	
M2G1D4	400	20	
M2G1D5	400	25	
M2G2D5	1000	25	
M2G3D5	3500	25	
M2G4D5	4000	25	
M2G1D5	400	25	concentration of PEG–INH (mg/ml)
M2G1D5P1	400	25	14
M2G1D5P2	400	25	28
M2G1D5P3	400	25	44
M2G1D5P4	400	25	59
M2G1D5P5	400	25	63
M2G1D5P6	400	25	79
M2G1D5P7	400	25	83
M2G1D5P8	400	25	100

*M2 stands for INH, for instance M2G1D1 is equivalent to INH-G1-D1. The concentration of PEG–INH (mg/ml) indicate the increasing concentration of M2G1D5 (INH-PEG (400 Da)-D5 (25 kGy)).

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Cytotoxic testing was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium (MTT, Sigma-Aldrich) reduction assay to determine the number of viable THP1 cells in culture [30]. The human acute monocytic leukemia cell line THP1 was acquired from the American Type Culture Collection (TIB202, ATCC). THP1 cells were grown in RPMI-1640 medium supplemented with 2-mM L-glutamine, 10-mM HEPES buffer, 1- mM sodium pyruvate (Lonza, CA, USA), 10% heat-inactivated fetal bovine serum (HycloneTM, Logan, USA), and 50-μM β-2-mercaptoethanol (Bio-Rad Laboratories, Berkeley, CA, USA). The cells were seeded in 48-well plates containing culture medium described above for 36 h at a temperature of 27°C until a density of 0.3×10^6 cells/well. THP-1 cells were differentiated into macrophage by exposing them to a 50- nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) solution for 3 d, as previously described [31].

The PEG–INH (339 to 5420 μg/ml) was added to the wells containing cells. Plates were incubated in an oven at 37°C under a 5% CO_2 atmosphere for 24h, and the cell viability was quantified. After this, the plate was centrifuged to eliminate the treatment, and immediately red phenol free RPMI fresh medium and 30- μl MTT (5 mg/ml) were added, and plates were incubated for 2 h more. Absorbance at 570 nm was read on an HT Multi-Mode Microplate Reader (Biotek, WA, USA).

3. Results and discussion

Fig. 1 shows the Raman spectrum of pure solid and dried isoniazid (Fig. 1a), aqueous polyethylene glycol (400 Da) (Fig. 1b), PEGylated isoniazid (Fig. 1c), and the effect of different doses on the synthesis of PEGylated isoniazid (Fig. 1d) [32]. As can be seen, the bands at 3307, 3115, and 3059 cm^{-1} belong to amine stretching vibration (ν(N—H)), and two signals of C—H asymmetric stretching (ν(C—H)) respectively (Fig. 1a). Moreover, the PEGylated drug (M2G1D1) showed a broad band within the range of 3000–3500 cm^{-1}, which is associated with the reaction of PEG with a primary or secondary amine group of isoniazid (Fig. 1c). Rather than a band at 1674 cm^{-1}, identified as carbonyl stretching of neat INH (ν(C═O), Fig. 1a), a small and weak band at 1667 cm^{-1} is observed which suggests the formation of amide groups (R_1CONR_2) (Fig. 1c) [33].

The comparison of the two spectra (M2/M2G1D1; Fig. 1a and c) showed a slight variation in the signals attributed to the heterocyclic structure, namely, ring C═C asymmetric stretching (1611 cm^{-1}), C—N stretching ($\approx$1337 cm^{-1}), H—N—H bending (1551 cm^{-1}), ring C—C—H asymmetric bending ($\approx$1192 cm^{-1}), and ring C—N—C asymmetric bending ($\approx$887 cm^{-1}). This result revealed that the aromatic ring was not affected in the radiochemical reaction. In addition, the overlapping of the band originally found at 1131 cm^{-1} (N—X stretching, X═NH$_2$ group) exhibited a possible reaction with PEG by primary amine [34].

The disappearance of the isoniazid C—C—H symmetric bending at 849 cm^{-1} with regard to the final product represent a probable distortion caused by PEGylation. Furthermore, the Raman of PEG was previously discussed (Fig. 1b) [28]. It was noted that the M2G1D1 spectrum does not contain PEG signals, which implies that both
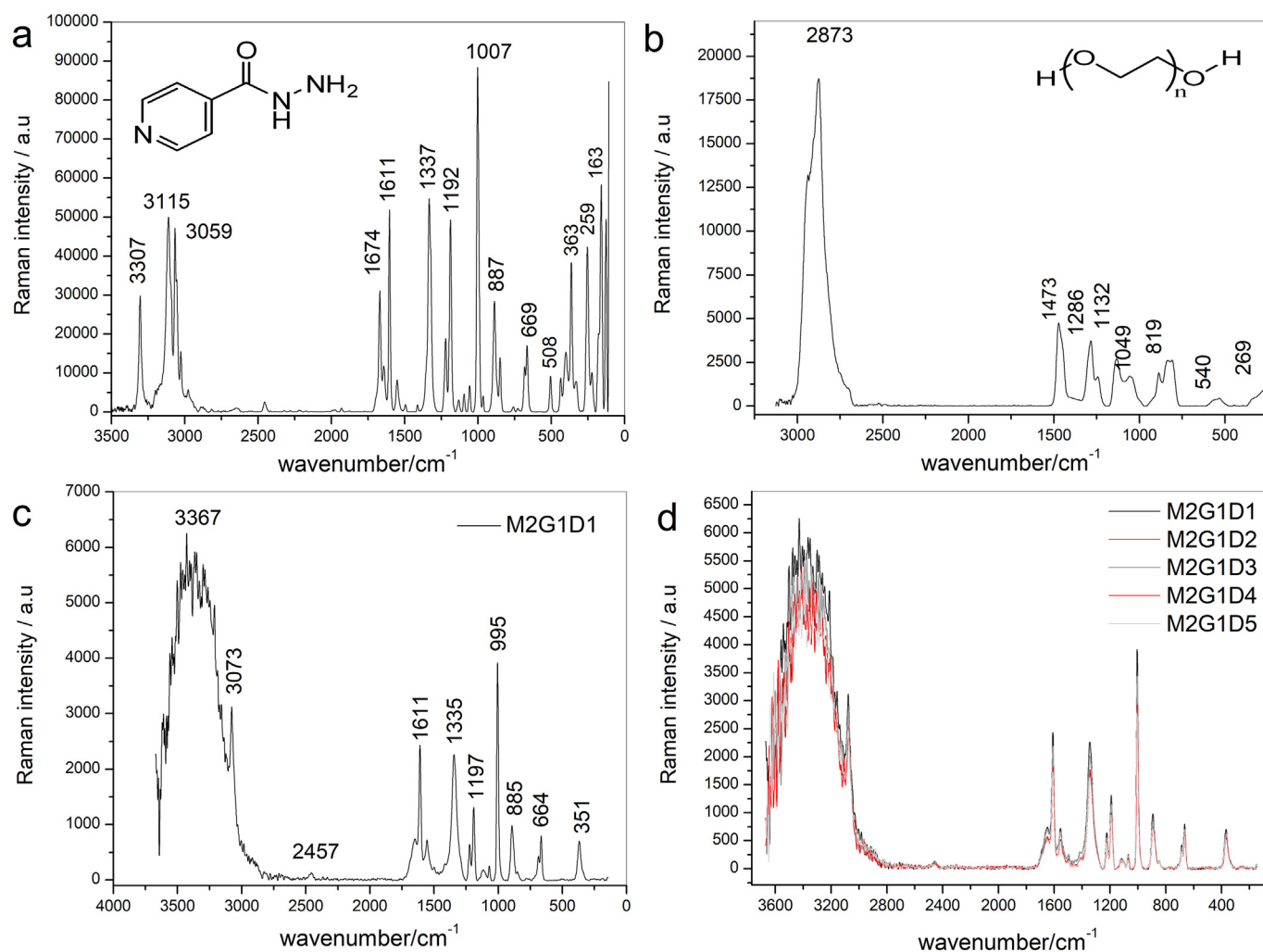

Fig. 1. (a) Raman spectrum of pure solid isoniazid. (b) Raman spectrum of aqueous polyethylene glycol (400 Da). (c) Raman spectrum of PEGylated isoniazid. (d) Effect of different doses on the synthesis of the PEG–INH.

molecules have reacted completely. The disappearance of the major part of the isoniazid fingerprint (100–600 cm^{-1}) is also observed, except for the band at 351 cm^{-1}. Finally, the spectra of PEGylated isoniazid obtained at increasing dose points towards no apparent differences between each other (Fig. 1d). The results support the successful synthesis of PEGylated isonicotinylhydrazide [35].

Fig. 2 shows the UV–visible spectra of (a) isoniazid and PEG-g-INH, (b) samples with different doses of gamma radiation, (c) increasing molecular weights of poly(ethylene glycol) in the graft reaction, and (d) increasing concentrations of M2G1D5. The isoniazid displays an ultraviolet maximum at roughly 263 nm [36]. The emergence of a new peak at 358 nm was ascribed to the synthesized PEGylated drug (Fig. 2a). It is believed that this weak absorbance is attributed to the additional transitions between non-bonding orbitals and π* orbitals of a carbonyl group formed as a consequence of grafting of PEG onto the isonicotinylhydrazide. Therefore, it makes sense to have the same trend for increasing gamma radiation doses and PEG molecular weights (Fig. 2b and c). In Fig. 2d, it also can be seen that there is a clear dependency between absorbance and PEG–INH concentration. Since there is no variation in pH (7.4), it is concluded that concentrations over 28 mg/ml (P2) are needed to exhibit steady signals at 358 nm, as evidence of successful synthesis of gamma-radiation-induced direct PEGylation of isoniazid.

Fig. 3 shows the proposed mechanism of PEGylated isoniazid by the simultaneous irradiation method. First, in the initiation step, the radicals of PEG and INH are formed. To simplify the mechanism, the radiolysis of water was omitted. On the one hand, PEG macroradical contains a carbonyl radical at the end of the molecule, while on the other side the drug has two alternatives, the radicals over the primary or secondary amine. Second, the INH radical react by radical coupling with PEG macroradicals to yield PEG-g-INH prodrug. From Raman results, it is suggested that PEGylation is more likely to occur by primary amine.

First, we evaluated the effect of the conjugate PEG–INH (M2G1D1) on *M. tuberculosis* H37Ra growth and determined that this new compound caused a dose-dependent decrease in bacterial growth at doses from 0.1 to 5.0 μg/ml when the bacterium was cultured in liquid medium (Fig. 4). The bactericidal effect of PEGylated INH increased significantly with respect to INH by itself. Interestingly, the conjugate INH–PEG inhibited the bacterial growth above 95% at a concentration of 0.1 μg/ml (0.25 μM) and more than 99% when the concentration was increased ten times. All synthesized products exhibited the same trend.

The increased bactericidal effect exhibited by the INH–PEG could be due to the conferred partial hydrophilicity by the PEG molecule grafted on it. This property could contribute to stability in the culture medium avoid its degradation in the aqueous medium. In fact, PEG chains
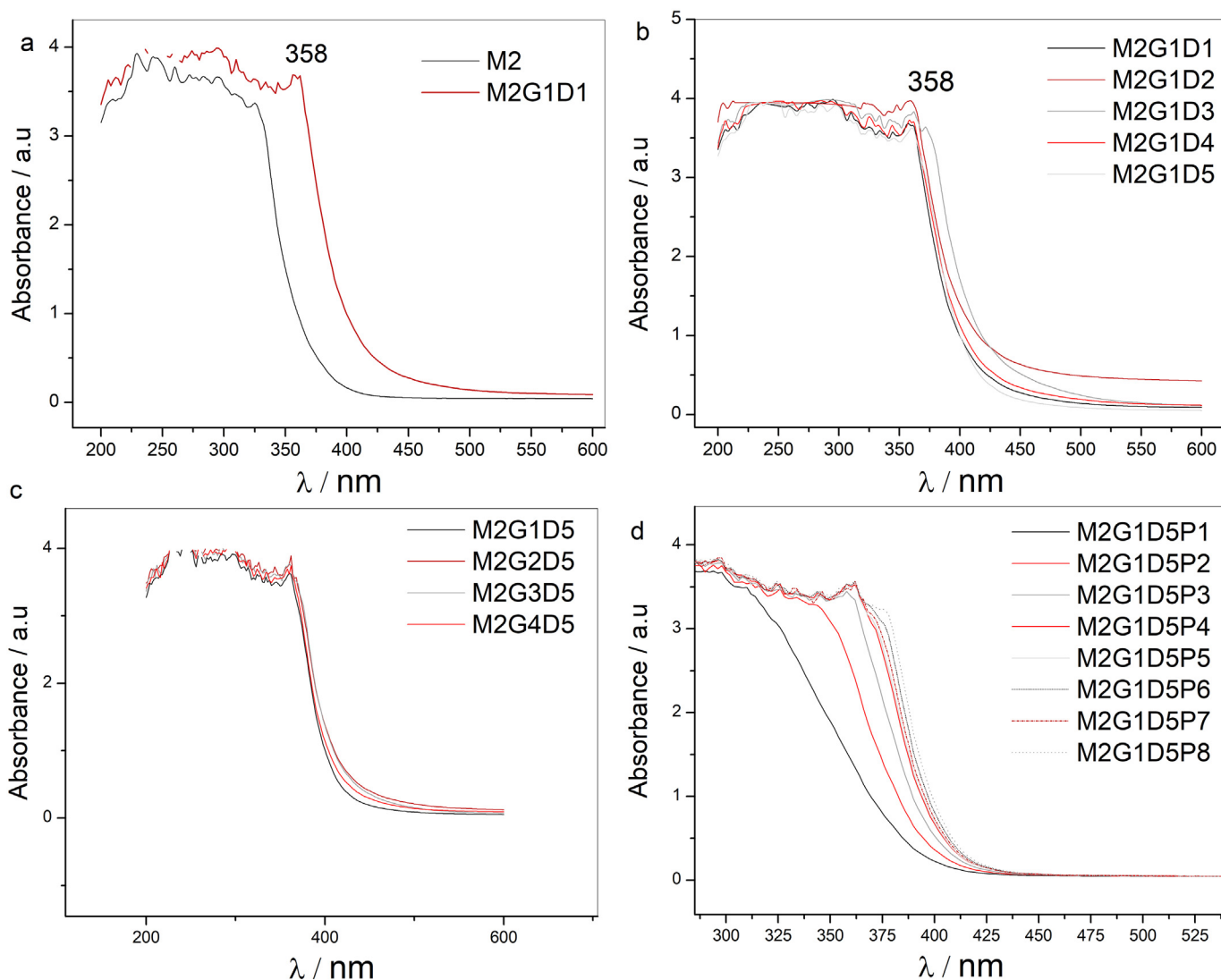

Fig. 2. (a) UV/Vis absorption spectra of isoniazid and PEGylated isoniazid. (b) UV/Vis study of the effect of different doses on the synthesis. (c) UV/Vis analysis of the effect of the use of increasing molecular weights of polyethylene glycol on the synthesis of PEG-g-INH. (d) UV/Vis survey of the effect of the use of increasing concentrations of PEGylated prodrug.
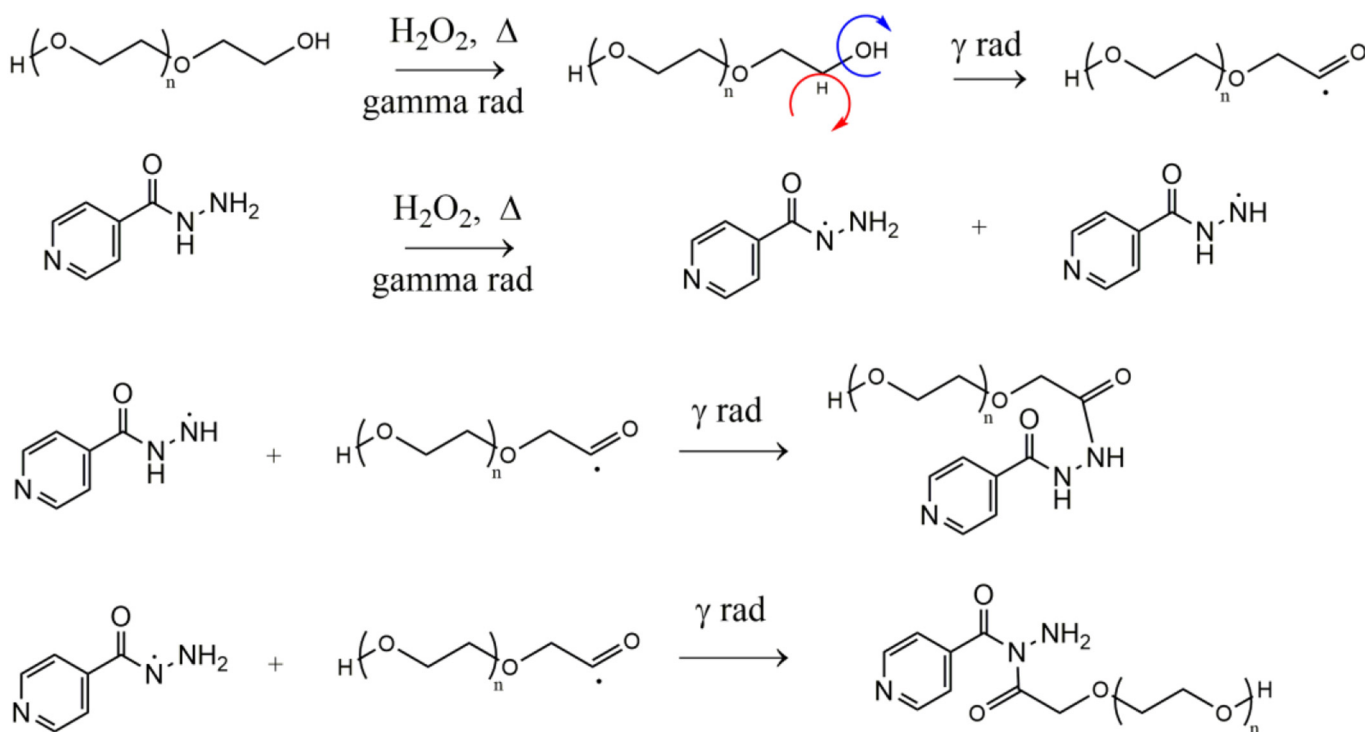
Fig. 3. Proposed mechanism of gamma radiation-induced PEGylated isoniazid.

confer more solubility because it can form a large number of H-bonds with water and increase the INH solubility and avoid the possible aggregation. These skills could enhance its bactericidal activity [37,38]. Despite of the limitations of this work, we were able to demonstrate that INH-PEGylated maintains its bactericidal activity. However, it is important to show that this activity is maintained in the infection context.

It is known that isoniazid can be susceptible to enzyme hydrolysis. However, PEG chains might protect isoniazid from the action of *N*-arylaminoacetyltransferases [14]. It has been recently demonstrated that the PEG attached to an L-asparaginase protects it from the hydrolysis by two serum proteases. After 84 h of contact with the proteases, the PEGylated L-asparaginase maintains its biological activity [39]. It is worth mentioning that the choice of H37Ra instead of using H37Rv is justified by the report of a similar response to anti-TB agents [40].

Regard to *in vitro* cytotoxicity, none of the tested concentrations of PEG–INH significantly decreased THP-1 cell viability after exposing the cells to the new drug for 24 h. Similar behavior was observed with the
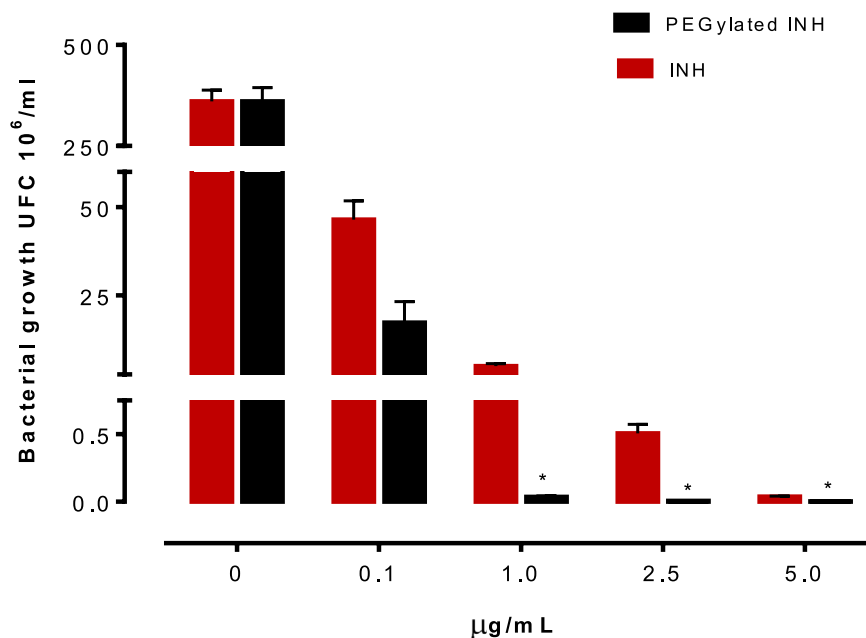

Fig. 4. Effect of PEGylated isoniazid on bacterial growth. Bacteria were cultivated in 7H9 Middlebrook medium supplemented with INH or PEG–INH for 7 d. After incubation, CFUs were quantified by counting the number of surviving bacteria for each concentration of compounds. Deviation and the statistical analyses were conducted using one-way analysis of variance (ANOVA) p values < 0.05 were considered as statistically significant. Data represent the mean ± SE (n = 4). *p < 0.05 compared to the culture with INH alone. PEG–INH concentration in μg/ml refers to INH equivalents.
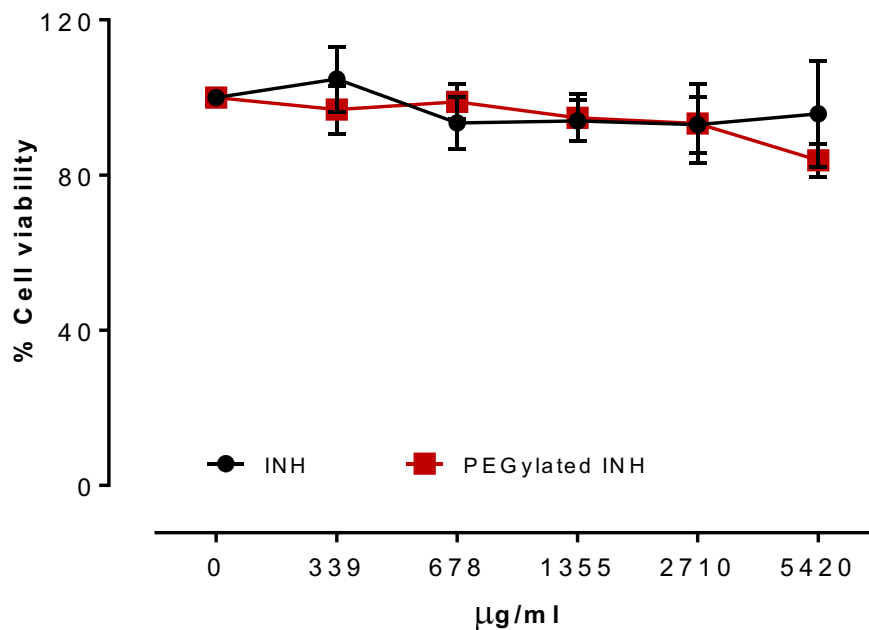
Fig. 5. Effect of PEG–INH on THP1 cell viability: The INH or PEG-g-INH (0–5420μg/ml) were added for 24h. PEG–INH concentration in μg/ml refers to INH equivalents. At the end of the exposition time, the media containing the compounds were replaced with fresh medium and MTT was added for 2 h. The number of viable cells is expressed as a percent of MTT reduction with respect to cell without treatment. The experiment was performed in triplicate.

sole isoniazid (Fig. 5). The fact that the new PEGylated drug did not kill THP-1 cells was expected as it is known that PEG is approved by the Food and Drug Administration (FDA). The use of THP-1 cells to evaluate the cytotoxicity of an anti-tubercular agent is thus justified by the following: MBT infection primes human alveolar macrophages [41], and THP-1 cells resemble primary monocytes-macrophages isolated from healthy donors, so they mimic macrophages [42]. It is commonly used to evaluate cytotoxicity [43]. The above suggests that THP1 cells represent a suitable model to prove the cytotoxicity of INH–PEG. It is important to mention that PEG might reduce, in some cases, the target drug uptake by the cells as claimed in some studies [44]. However, recent investigations have demonstrated that particles (80×320 nm and 1.5 μm) modified with PEG, which are larger in size as compared to INH-PEG, were still observed in lung macrophages after 28 d of internalization. Authors suggest that these particles are important candidates for diseases targeting macrophages in the lung, such as tuberculosis [45]. Nonetheless, evaluation of the INH-PEG uptake by macrophages *in vitro* and *in vivo* will be addressed in the next steps of this research.

There are serious shortcomings found in the use of small pharmaceuticals, namely the rapid excretion, the low solubility and the untargeted biodistribution, which can be overcome by PEGylation. Small drugs covalently bonding with PEG can also address adequate drug delivery, minimize toxicity problems, and increase macromolecular activity. However, these drug–polymer conjugates are usually limited by the low loading of poly(ethylene glycol). We have attempted to address this knowledge gap by directly linking PEG to the neat drug. The most important PEG–INH advantages would be the increase of biological activity alleged by the ability to overcome the impermeability of MTB and the future increase of oral bioavailability.

4. Conclusions

Gamma-radiation induced PEGylated isoniazid was synthesized by the simultaneous irradiation method. The PEG–INH conjugate was structurally characterized by Raman and Ultraviolet–visible spectrophotometry. The evidence obtained by spectroscopy suggests that PEGylation of the tuberculostatic agent was successfully achieved. The most likely reaction is the grafting of PEG onto the

isonicotinylhydrazide primary amine group. The advantage of direct covalent bonding of poly(ethylene glycol) onto the isoniazid is the high loading of the polymer and the easy and pure synthesis of a prodrug.

On biological *in vitro* evaluations, the PEGylated isoniazid showed greater anti-mycobacterial activity than unmodified INH. The bacterial growth was inhibited above 95% at a concentration of 0.25 μM. A clear additional advantage of PEGylation is the increase in bioavailability of the isonicotinylhydrazide. The toxicity of both pharmaceuticals is similar. The THP-1 cell viability was not meaningfully decreased by both drugs, thus evidencing the significant clinical potential of the novel derivative.

Further research will include evaluation of this innovative prodrug agent against other *M. tuberculosis* strains and *in vivo* studies, including evaluation of INH-PEG uptake by lung macrophages. However, the new conjugate can be considered as a promising anti-tuberculosis agent as it showed high anti-mycobacterial potency and low cytotoxicity.

Conflict of interest

The authors of this manuscript declare no conflict of interests.

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