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ORIGINAL PAPER

Reduction of nitroblue tetrazolium to formazan by folic acid

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The reduction of nitroblue tetrazolium (NBT) to formazan by folic acid, *N*-(4-aminobenzoyl) glutamic acid, and other amino acids was studied in this paper. The reduction involves only one of the two tetrazolium rings of NBT. The reaction is considerably more rapid with folic acid and *N*-(4-aminobenzoyl) glutamic acid than with the other amino acids under study. The electron donor moiety appears to be the carboxylic acid in the alpha position. *N*-ethyl-*N*′(3-dimethylaminopropyl) carbodiimide notably increases the rate of the reaction and promotes the reduction of both tetrazolium rings.
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Keywords: nitroblue tetrazolium, formazan, carbodiimide, folic acid, amino acids

Introduction

Many tetrazolium salts, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS), 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolium)-1,3-benzene disulphonate (WST-1), and 2,3-bis-(2-methoxy-4-nitro-5-suphophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT), are used to investigate cell proliferation and viability in colorimetric assays based on reductive cleavage of the tetrazolium ring by metabolically active cells, resulting in the production of water-soluble or water-insoluble dark-blue formazans (Berridge et al., 2005). Furthermore, 2,2′-bis(4-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4'-diphenylene) ditetrazolium (nitroblue tetrazolium, NBT) can be reduced to water-insoluble monoformazan or diformazan by superoxide anions (Fig. 1); for this reason, it is widely used in colorimetric assays designed to measure the superoxide ions released during the activation of phagocytic cells (granulocytes, macrophages) (Berridge et al., 2005).

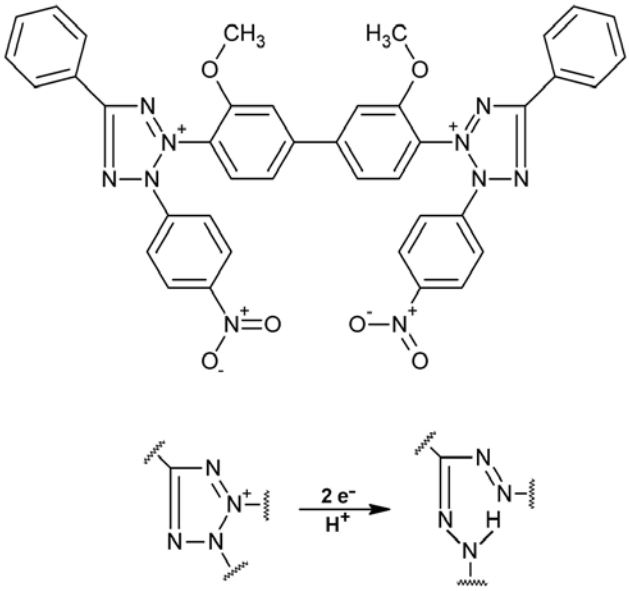

Fig. 1. Structure of NBT and reduction of tetrazolium ring to formazan.

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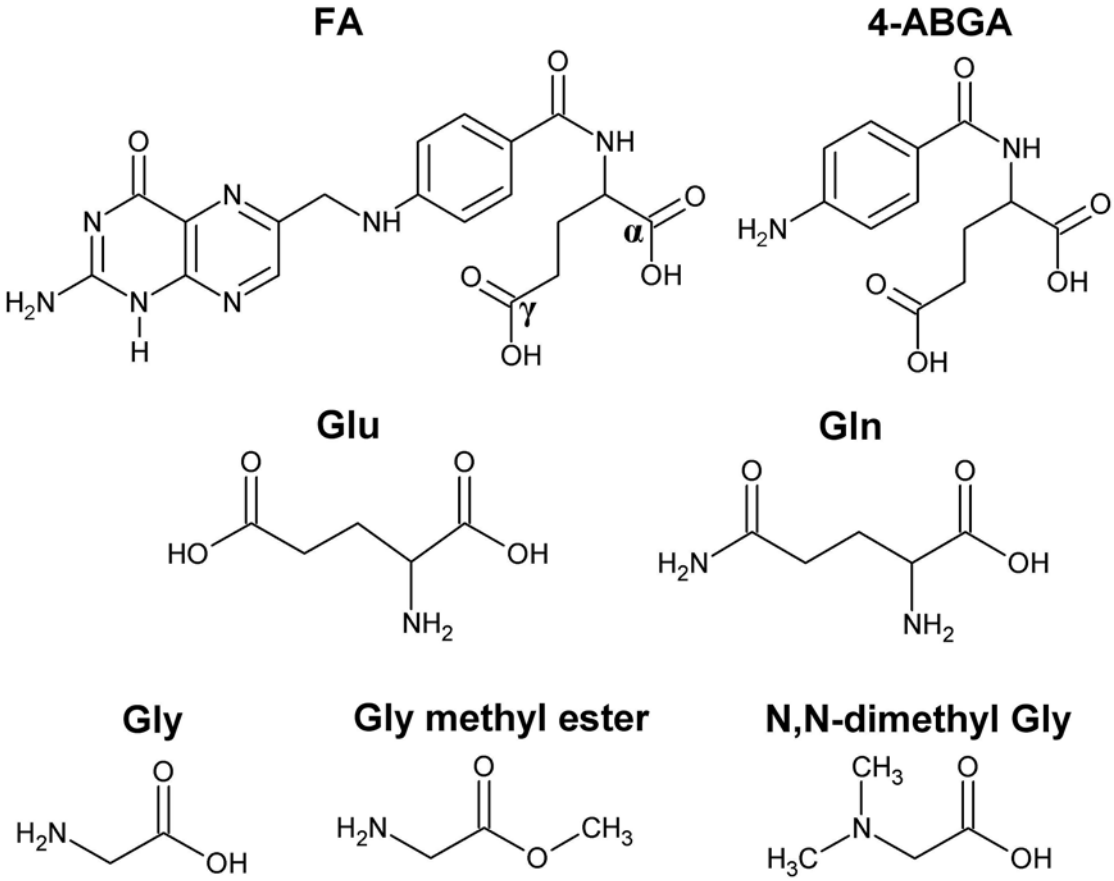

Fig. 2. Structures of molecules tested as NBT-reducing agents: folic acid (FA), *N*-(4-aminoenzoyl) glutamic acid (4-ABGA), glutamic acid (Glu), glutamine (Gln), glycine (Gly), glycine methyl ester (Gly methyl ester), *N*,*N*-dimethyl glycine (N,N-dimethyl Gly).

Any extracellular molecules that act as electron-donors can potentially reduce the tetrazolium ring and significantly increase the signal of the assay, yielding a false positive result. Many components of cell culture media, such as antioxidants, vitamins, and serum proteins (albumin), can react with MTT and XTT (Natarajan et al., 2000; Chakrabarti et al., 2001; Bernhard et al., 2003; Funk et al., 2007). Numerous compounds, such as ketones, α-hydroxyketones, thiols, quinines, and polyhydric phenols, can also reduce NBT to formazan (Manni & Sinsheimer, 1961; Sinsheimer & Salim, 1965; Graham et al., 1975; Oteiza et al., 1977).

This study found that folic acid (FA; Fig. 2) can reduce the NBT to formazan.

Experimental

The main reagents used were purchased from Sigma–Aldrich (Italy). 1 mM stock solution of NBT (dichloride salt) in absolute ethanol and 20 mM aqueous stock solution of FA (disodium salt) were prepared. FA could only be brought into solution by salification with a stoichiometric quantity of NaOH, because the free acid is not soluble in water or in the majority of commonly used organic solvents. Mixtures of NBT and FA, each at a final concentration of 0.5 mM, were prepared using ethanol/water ($\varphi_r = 1 : 1$), and incubated at 25 ℃ in the dark. After 72 h, 0.5 volume (with respect to the sample volume) of absolute ethanol was added, the samples were sonicated to completely solubilise the formazan and then analysed by TLC on silica gel 60 plates (Merck, Germany) by elution with 1-butanol, acetic acid (100 %), and water, ($\varphi_r = 78 : 5 : 17$) as described in the literature (Altman, 1974). Fig. 3a shows a typical TLC elution of a sample, where the purple spot corresponds to monoformazan, as indicated by comparison with a standard mixture of monoformazan and diformazan loaded in the adjacent lane. To further confirm its identity, the spot was scratched from the plate, extracted with absolute ethanol and analysed by electrospray ionisation-mass spectrometry (ESI-MS, LCQ FLEET ion trap mass spectrometer, Thermo Fisher Scientific, USA). The positive ion mass spectra indicated a molecular mass of 748 Da, consistent with the monoformazan structure (Fig. 3b).

To understand which of the many functional moieties of FA was involved in this reaction, and to characterise the underlying mechanism of the reaction, sev-

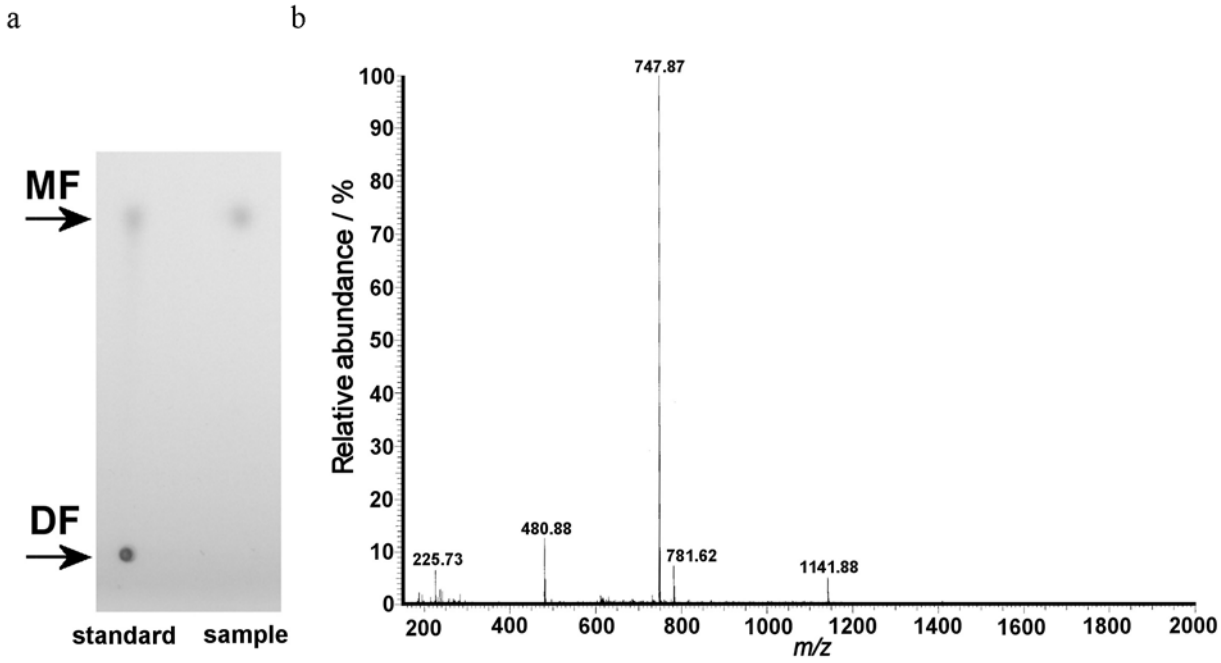

Fig. 3. TLC for detection of formazans (a). A mixture of monoformazan (MF) and diformazan (DF) as reference standard and the sample prepared by incubation of NBT with FA were loaded on the plate. The positive ion mass spectra (ESI-MS) of the monoformazan purified by TLC (b).

eral other molecules with structures and functional groups similar to those of FA were tested for their ability to reduce NBT: 4-ABGA, Glu, Gln, Gly, Gly methyl ester, N,N-dimethyl Gly (Fig. 2).

The reaction mixtures were prepared as follows. For the solubilisation of FA, 20 mM stock solutions of the other derivatives, irrespective of their hydrosolubility, were prepared in water as sodium salts, with a stoichiometric quantity of NaOH. The final concentration of NBT and of each compound tested was 0.5 mM. After incubation in the dark at 25 °C for 144 h, 0.5 volume of absolute ethanol was added to the samples, which were then sonicated. 100 μL of the sample was then used for quantitative determination of the formazan content by reading the absorbance at 550 nm, using a 96-well microtitre plate reader (Tecan, Switzerland). Fig. 4 shows the results obtained. The sample containing Gly methyl ester did not show any appreciable absorbance reading, indicating the absence of formazan. Conversely, all the other molecules exhibited a significant production of formazan, albeit with different intensities: 4-ABGA exhibited a reactivity similar to that of FA, which was approximately 60 % higher than those exhibited by Glu, Gln, Gly, and N,N-dimethyl Gly. For these other compounds, TLC analysis also confirmed formation of the monoformazan only (not shown).

Results and discussion

Taking the structural differences of the various compounds into consideration, it is worth noting that

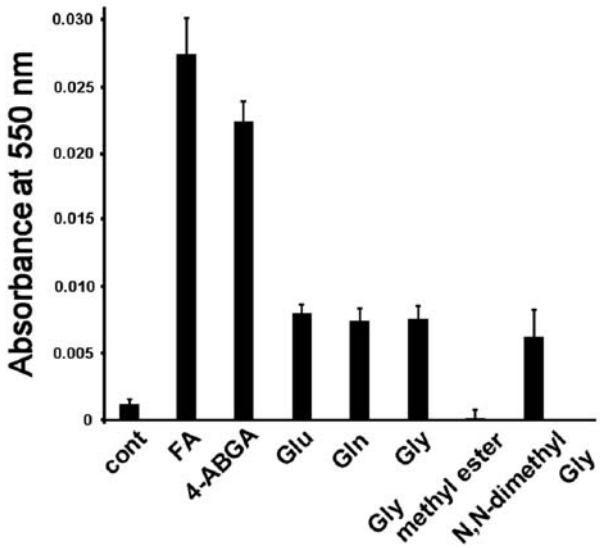

Fig. 4. Reduction of NBT by different compounds; in the y-axis, absorbance at 550 nm is reported: cont – NBT only. Values are the mean ± SD of three experiments.

4-ABGA exhibited a reducing capacity only slightly lower than that of FA, despite the absence of the pterin moiety in its structure. Glu, an amino acid in which the 4-aminobenzoyl group is also absent, had a reactivity considerably diminished but still present. Glu presents three possible reactive sites: α-amino, α-carboxyl, and γ-carboxyl groups. Gln, a derivative of Glu in which the γ-carboxylic acid is blocked by an amide bond, displayed a reactivity level superimpos-

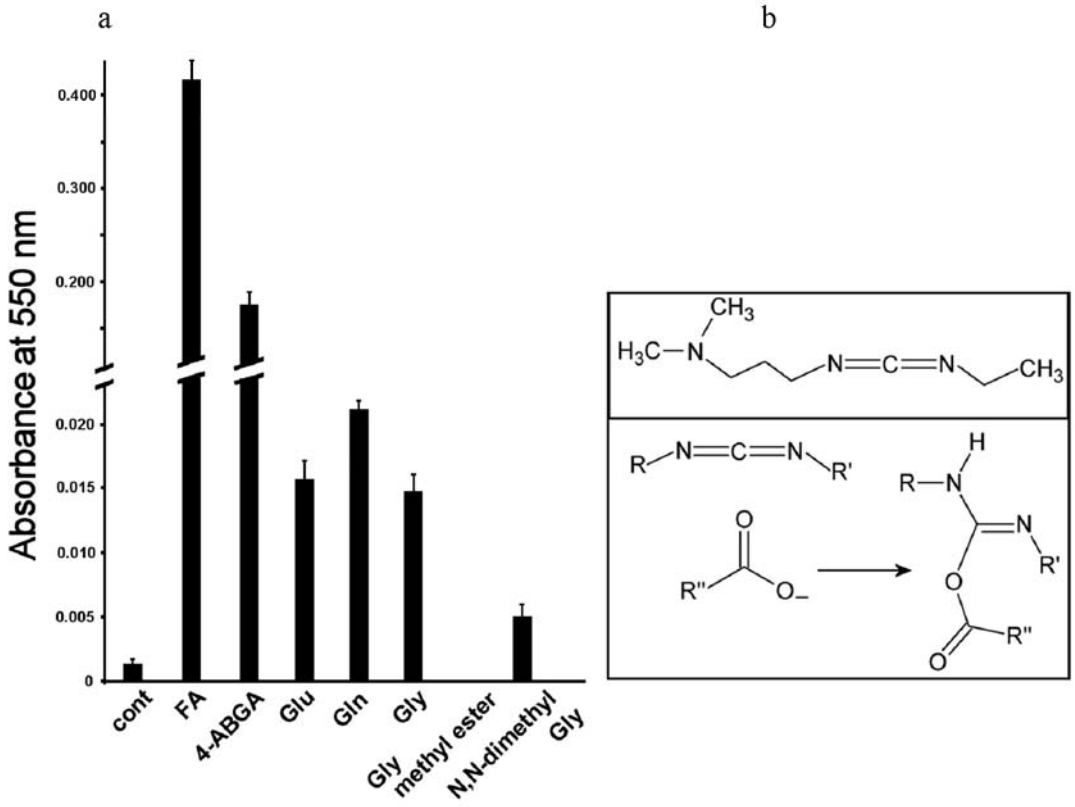

Fig. 5. Reduction of NBT by different compounds in the presence of EDC (a). In the y-axis, the absorbance at 550 nm is reported; cont – mix of NBT and EDC. The values are the mean ± SD of three experiments. The structure of EDC and the scheme of reaction with carboxylic acid are represented (b).

able to that of Glu. On this basis, it was hypothesised that the γ-carboxyl group is not involved in the reaction.

In addition, Gly, the simplest amino acid, endowed with α-amino and α-carboxyl groups only, had a reactivity superimposable to that of Glu and Gln. By blocking the α-amino moiety of Gly by methylation (N,N-dimethyl Gly), the formazan production remained stable, but after blocking the α-carboxylic acid by methylation (Gly methyl ester), the reactivity was suppressed, becoming similar to the control (cont; Fig. 4). These results strongly suggest that the moiety implicated in the reduction of NBT by FA is the α-carboxylic acid.

Also tested was the reduction of NBT by FA and by the other compounds in the presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC), a coupling agent that reacts selectively with carboxylic acids forming an O—C bond. The literature reveals that both carboxyl groups of FA can react with carbodiimides and other coupling agents, although the reaction favours γ-carboxyl group (Wang et al., 1996; Gabizon et al., 1999; Wei et al., 2005; Viola-Villegas et al., 2008). For the experiments, 5 mM of stock solution of EDC hydrochloride was prepared in water and used immediately at a final concentration of 0.5 mM. All the other reagents were also used at a final concentration of 0.5 mM. The reaction mixtures, after incubation in the dark at 25 ℃ for 144 h were supplemented with 0.5 volume of absolute ethanol and homogenised by sonication prior to reading the absorbance at 550 nm, by a 96-well microtitre plate reader (100 μL aliquots). Fig. 5 shows the results obtained. EDC considerably increased the rate of the reaction for most compounds, except for N,N-dimethyl Gly, where the reactivity was unchanged, and for Gly methyl ester, which remained unreactive. The increase in formazan production was fifteen-fold for the FA sample, nine-fold for 4-ABGA, and approximately two-fold for Glu, Gln, and Gly. TLC analysis of FA and 4-ABGA samples revealed the production of both monoformazan and diformazan in approximately comparable amounts (not shown). Furthermore, a moderate increment in the rate of NBT reduction by FA was observed in the presence of the coupling agent *N*-hydroxysuccinimide (not shown).

FA and the other amino acids tested in this study have different characteristics concerning their ionisable groups (amino and carboxylic) that could influence the pH value of the mixture and, potentially, modify the rate of the reaction. For this reason, the initial pH of the different samples prepared with EDC was measured (pH-meter: InLab Expert Pro, Mettler-Toledo, USA). The values ranged from pH 6 to pH 9 (FA, pH 7.3; 4-ABGA, pH 8.9; Glu, pH 8.9; Gln, pH 8.5; Gly, pH 8.8; Gly methyl ester, pH 6.0; N,N-

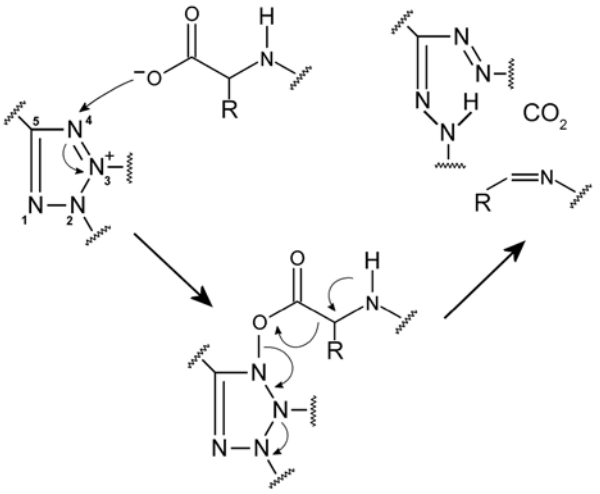

Fig. 6. Mechanism proposed for reduction of NBT by FA and related compounds.

dimethyl Gly, pH 8.7). To assay the potential effect of the pH on the reaction, a set of samples containing NBT, FA, and EDC (each at the final concentration of 0.5 mM) at different pH values (pH 6, pH 7, pH 8, and pH 9) was prepared, using sodium phosphate as buffer (5 mM, final concentration). The samples were incubated for 72 h and processed for the measurement of formazan production as detailed above. The amounts of formazan produced did not differ substantially in the reactions carried out at pH 7, pH 8, and pH 9. Conversely, the sample at pH 6 gave a yield of formazan 25 % higher than the other buffered samples (Fig. S1 in Supplementary materials). For this reason, the different reactivity observed in unbuffered samples containing FA and the other amino acids may be excluded as being due to the different pH. Furthermore, the lack of reactivity in the sample containing Gly methyl ester which, by contrast, has a pH value that could favour the reaction, corroborates involvement of the carboxylic group in the reaction mechanism.

It is hypothesised that the reaction occurs as nucleophilic attack by the carboxylic oxygen on the nitrogen in position four of the tetrazolium ring, the electron acceptor proposed for the reduction of tetrazolium by hydroxy ketones (Oteiza et al., 1977). The following step could be the break of the O—N bond with the donation of the electrons to the tetrazolium nitrogen, followed by the break of the bond between the nitrogen atoms in positions two and three of the ring, with the formation of formazan. The probable rearrangement of FA (and of the other compounds) could be the formation of a Schiff base between the α-carbon and nitrogen, followed by the release of carbon dioxide, as represented in Fig. 6.

FA and 4-ABGA have the α-amino group engaged in an amide bond, a condition that generally makes the nitrogen atom electron-poor due to its involvement in the resonance with the carbonyl group of the amide. This would not favour the reaction of the α-carboxyl group with NBT as much as hypothesised. However, the reaction may still be promoted, thanks to electronic effects induced by the 4-aminobenzoyl moiety present in FA and 4-ABGA. In effect, the resonance between the amino group in the *para* position of the aromatic ring and the carbonyl group of the amide does not involve the nitrogen atom of the amide. This could confer on it an electron-donor character sufficiently pronounced to promote the reaction of the α-carboxyl group, contrary to what would occur with other aromatic or aliphatic amides. The α-nitrogen could equally act as an electron-donor sufficiently to promote the formation of a Schiff base during the rearrangement of FA. These electronic effects may also partially explain the consistent differences in kinetics observed between the simple amino acids and the compound in which the α-amino group is engaged in an amide bond.

The increased rate of the reaction in the presence of EDC could be due to the coupling of carbodiimide with the α-carboxyl group, which may promote the nucleophilic attack on the tetrazolium ring, further supporting the evidence concerning the involvement of a carboxyl group in the mechanism of the reaction. It cannot be excluded that EDC also reacts with the γ-carboxyl group, even at a higher rate than with the α-carboxyl group, as already known from the literature; however, this event could not be detected in the experimental system in this study, because it would not be productive in terms of NBT reduction.

The presumed mechanism is also partially supported by ESI-MS analysis of the reaction mixture prepared with FA in the presence of EDC, in which a molecule with a molecular mass compatible with the carboxylated form of NBT is clearly detectable, as a possible intermediate product of the reaction (Fig. S2 in the Supplementary materials). Although the corresponding Schiff base derived from the rearrangement of FA was not detected, it should be noted that FA was also not detected by ESI-MS analysis, even though it was still present in significant amounts (at least 125 μM) at the end of the incubation, as determined by RP-HPLC (data not shown). This phenomenon could be due to the high propensity of FA and of its derivative(s), to fragment during ionisation, generating a complicated and often non-reproducible ionisation pattern, and to form neutral salts or zwitterion species which suppress the MS signal.

The results given in this paper indicate preliminary data concerning a new method for the reduction of NBT to formazans. It cannot be excluded that other tetrazolium salts may be reduced in the same manner.

The growth media in common use for the in vitro culture of eukaryotic cells have a total concentration of free amino acids in the millimolar range. On the basis of the results given in this paper, these relatively high levels of amino acids in a cellular sample could

interfere with metabolic assays based on tetrazolium reduction, affording false positive responses. FA is also present in cell culture media although at nanomolar levels, too low to significantly interfere with the assay. However, in recent years, many FA-derivatives have been designed and in vitro tested for the selective delivery of therapeutic and diagnostic molecules into malignant cells that express FA-receptors at higher levels than normal cells (Low & Kularante, 2009). Furthermore, FA-derived molecules such as amethopterin (methotrexate) and aminopterin are proposed for the treatment of cancer, auto-immune diseases, and infections due to their inhibitory effects on FA metabolism (Takimoto, 1996). These FA-based agents are preliminarily tested with cells grown in vitro to assess their efficacy, safety, and biological properties, at millimolar concentrations (Walker et al., 2000), thus potentially interfering with tetrazolium-based colorimetric assays.

FA and its metabolites are important biological components of the cell, essential for several processes such as DNA and amino acid synthesis, and their depletion induces many cellular disorders and diseases. Hence, the reactivity with FA could be the cause of the toxicity of tetrazolium compounds found in several studies (Nineham, 1955). This phenomenon requires further investigation, to clarify the mechanism of the interaction of tetrazolium compounds with biological systems.

Conclusions

The reduction of NBT to formazan by FA and other amino acids is described in this article. The reduction, which involves only one of the two tetrazolium rings of NBT, is considerably more rapid with folic acid and *N*-(4-aminobenzoyl) glutamic acid than with the other amino acids examined. The electron-donor moiety appears to be the carboxylic acid in the alpha position. The relatively high concentrations (millimolar) of amino acids present in cell samples and in vitro biological systems such as cell culture media could produce false positive results when metabolic assays based on tetrazolium reduction are used. Although FA is present in biological fluids at submicromolar concentrations, many FA-derivatives are used in vitro as therapeutic and diagnostic molecules. Accordingly, attention should be paid to the potential reduction of tetrazolium salts when high doses of amino acids, FA and its derivatives are present in cellular samples.

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