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Synthesis of F-18 labeled resazurin by direct electrophilic fluorination

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Abstract

We present the synthesis and characterization of F18-labeled fluorinated derivatives of resazurin, a probe for cell viability. The compounds were prepared by direct fluorination of resazurin with diluted [F18]-F₂ gas under acidic conditions. The fluorination occurs into the *ortho*-positions to the hydroxyl group producing various mono-, di-, and trifluorinated derivatives. The properties of the fluorinated resazurins are similar to the parent compound with the addition of fluorine leading to decreased pK_a values and a bathochromic shift of the absorption maxima. The fluorinated resazurin derivatives can be used as probes for observation of cell viability in various cells, tissues and organs using a combination of positron emission tomography and direct optical imaging of Cerenkov luminescence.

Keywords

F-18; electrophilic fluorination; resazurin; resorufin; Cerenkov luminescence

Introduction

Labeling of complex organic molecules with the positron emitting isotope ¹⁸F by electrophilic fluorination with [¹⁸F]-F₂ gas is a relatively rarely used method, because this reaction usually results in non-specific decomposition of the target reagent [1]. Recently, we found that dilute fluorine gas can be used in acidic conditions for introduction of the ¹⁸F into molecules of various triarylmethane pH indicators with reasonable (5–10%) yields [2–4]. Our interest in this kind of compounds is driven not only by their potential application as positron emission tomography (PET) markers, but also because the high energy positrons from ¹⁸F-radioactive decay emit visible Cerenkov light, which can be selectively quenched by the colored carrier molecule. We have previously shown that a combination of PET and optical imaging of ¹⁸F-labeled pH indicators could be used for *in vivo* measurement of the hydrogen ion concentration in mice [5].

In this paper, we report the application of the method of ^{18}F electrophilic fluorination with ^{18}F - F_2 gas to another type of aromatic compounds, resazurin (7-hydroxy-3*H*-phenoxazin-3-one 10-oxide, also known as Alamar Blue). This compound is widely used as a marker for cell viability and aerobic respiration [6]. Resazurin (which is blue and very weakly fluorescent under neutral conditions) is reduced in viable cells into resorufin (pink and highly fluorescent), providing a method for optical detection of cell presence and their metabolic activity. The introduction of an intramolecular source of Cerenkov light (^{18}F) into the reporter molecule will allow application of the probe for *in vitro* and *in vivo* cell viability detection.

Materials and Methods

All reagents were purchased from Sigma-Aldrich. Labeling of the molecules with ^{18}F was performed at Cyclotron Facility in the Department of Radiology at the University of Pennsylvania using a custom-made electrophilic fluorination unit. ^{18}F - F_2 gas was prepared by the reaction $^{20}\text{Ne}(d,\alpha)^{18}\text{F}$ using an IBA 18/9 Cyclone accelerator. The labeling reaction was performed by bubbling ^{18}F - F_2 gas (0.1% ^{18}F - F_2 in bulk Ne with a total activity of 100–200 mCi in 30 micromoles of ^{19}F carrier) for 5 minutes through a freshly prepared solution of the sodium salt of resazurin (3–9 mg) in 10 mL of acetic acid (2 mg/mL) or resorufin (3–5 mg) in 5 mL of trifluoroacetic acid. The reaction mixture was evaporated under vacuum at 120°C, re-dissolved in 2 mL of water, and injected onto a semi-preparative HPLC column (Phenomenex, Synergi 4 μm Hydro-RP 80 Å 10 mm $\times$ 250 mm). The elution buffer was 26% ethanol-water for resazurin and 17% ethanol and 5 mM ammonium-phosphate buffer (pH=7.6) for resorufin at a rate of 3 mL/min with simultaneous detection of radioactivity and UV absorption at 575 nm. Under these conditions elution times for the products of interest were in the range of 25–40 min providing sufficient peak separation without significant decay of the radioactive label.

Separation of the individual compounds was performed on the basis of the radioactivity peaks and UV signals. Individual fractions were collected and analyzed by analytical HPLC, mass spectrometry, NMR, UV-VIS and fluorescent spectroscopy. The presence of individual components in the fractions was confirmed by analytical HPLC performed with a 4.6 mm $\times$ 250 mm C-18 column and 10 mM H_3PO_4 /50% methanol buffer for resazurin and 5 mM ammonium-phosphate buffer (pH=7.6)/50% methanol for resorufin derivatives.

Incorporation of the fluorine atoms into the indicator molecule was confirmed using a Thermo Fisher Scientific (Bremen, Germany) Orbitrap Exactive mass spectrometer. The molecular mass of the products was determined after complete decay of the radioactive label, because the relative amount of ^{18}F in the radioactive ^{18}F - F_2 gas was very small (about 10^{-5} mole fraction) and would not give rise to any distinct signal.

The number of fluorine atoms in the molecule was confirmed by comparison of the ratio of the sample radioactivity to its optical absorption. For samples from the same preparation this ratio is proportional to the number of incorporated fluorine atoms, providing a way to confirm the data obtained by mass spectrometry.

The position of the fluorination in the molecules was determined from ^1H and ^{19}F NMR spectra obtained for basic (blue) forms of the indicators on a Bruker DMX-360 spectrometer using deuterated water as a solvent. Decoupling experiments were carried out with the set parameters $\text{O1} = 4.5$ ppm, $\text{O2} = -164$ ppm for $^1\text{H} \{^{19}\text{F}\}$ and $\text{O1} = -100$ ppm, $\text{O2} = 6.95$ ppm for $^{19}\text{F} \{^1\text{H}\}$, respectively.

The spectral properties and pK_a values of resazurin, resorufin and its fluorinated derivatives were analyzed by UV-vis spectroscopy. The pH dependence of the absorption of their basic forms was measured on a SpectraMax M5 (Molecular Devices) spectrophotometer using citrate/phosphate buffer over the pH range 2–8. The samples were diluted in 0.1 M trisodium citrate and 0.1 M phosphate and were titrated by addition of 1/100 volume portions of 1 M HCl with pH and spectral measurement after each addition. The titration curve ($A_{\lambda_{\text{max}}}$ vs. pH) was fit by the equation $A_{\lambda_{\text{max}}} = a/(K_a + [H_3O^+]) + b$ to determine the K_a value. The titration curves for resazurin derivatives presented in Fig.2, the data for pK_a and λ_{max} are summarized in Table 1.

Results

The reaction of diluted $[^{18}\text{F}]\text{-F}_2$ gas with a solution of resazurin in acetic acid caused stepwise introduction of fluorine atoms into the target molecule. Separation of the reaction mixture by HPLC showed the presence of non-reacted resazurin and four major radioactive products. Based on mass-spectrometric data and the radioactivity to absorption ratio, these products were characterized as monofluoresazurin (MFRA), two different isomers of difluoresazurin (DFRA), and trifluoresazurin (TFRA; the structure of the major products is shown on Figure 1).

The yield of the products was determined for varying amounts of resazurin reacting with the same amount of fluorine (30 μmol) following electrophilic labeling with $[^{18}\text{F}]\text{-F}_2$ gas [4]. In the range of 10–35 μmol resazurin, the amounts of fluorinated products were essentially the same, indicating 3:1 stoichiometry of the reaction. The yield of the products was determined as the ratio to the amount of fluorine gas, which is a critical parameter for incorporation of F-18 into radiolabeled probes. We determined the total chemical yields of the products ($n=12$) as $2.5 \pm 0.2\%$ for monofluoresazurins, $2.3 \pm 0.1\%$ for difluoresazurins, and $1.2 \pm 0.1\%$ for trifluoresazurin. Radiochemical yields of the reaction products were essentially the same for monofluorinated products, doubling and tripling for di- and trifluorinated derivatives. Despite relatively low overall yields of fluorinated products, up to 10 mCi of radiolabeled probe can be produced, which is enough for application in any biological object.

Further structural characterization of the products was performed by ^1H and ^{19}F NMR. Proton NMR of the monofluorinated product demonstrated that it contains two isomers, obtained from introduction of the fluorine atom into *ortho*-positions to phenol group. The NMR spectrum of resazurin under basic conditions is relatively simple and contains three signals from protons at carbon atoms 1 and 8 (δ , 7.67 ppm), 2 and 7 (δ , 6.53 ppm), and 4 and 5 (δ , 6.13 ppm; the assignment of the atoms is presented on Figure 1). Introduction of a fluorine atom into one of the aromatic rings causes a downfield shift of the proton signals

from adjacent rings by 0.3 – 0.5 ppm (δ , from 6.13 to 6.61 ppm for H₅, from 6.53 to 6.86 ppm for H₇; and from 7.67 to 8.11 ppm for H₈), regardless of the position of fluorination. NMR signals from the protons adjacent to the fluorine atoms undergo both a downfield shift and display coupling to the fluorine atom, making the spectrum quite complicated. A fluorine atom in position 4 shifts ¹H signals by ~0.3 ppm: from 7.67 to 7.95 ppm in the *para*-position (H₁'), and from 6.53 to 6.89 ppm in the *meta*-position (H₂'); the latter signal is coupled with fluorine. A fluorine atom in position 2 causes a similar (~0.3 ppm) downfield shift from 6.13 to 6.47 ppm of the signal of the proton in the *meta*-position (H₄'), and a much weaker downfield shift (only by 0.07 ppm from 7.67 ppm to 7.74 ppm) for the *ortho*-H₁; both of these signals are coupled with the fluorine atom. Decoupling experiments eliminated the splitting of the last three signals from the ¹⁹F atom, confirming the assignment presented above. ¹⁹F NMR shows the presence of a signal with $\delta = -163.64$ ppm, which corresponds to a fluorine atom in the *ortho*-position relative to the phenol group [7] and can be attributed to both 2- and 4-MFRA. The signal is broad, suggesting an overlap of signals with different couplings to protons in 4-MFRA and 2-MFRA. We were not able to separate the two isomers of the MFRA by varying the HPLC column material and separation conditions.

Comparison of the integral intensity of the proton NMR signals suggests, that about 80% of the mixture is represented by 4-MFRA (total yield 2%), and the remaining 20% is 2-MFRA (total yield 0.5%). These results correlate with the values of the chemical shift of the NMR signals for the corresponding protons. Position 4 is the most preferable site of electrophilic attack, because the hydrogen atom there has the lowest chemical shift on ¹H NMR (δ , 6.13 ppm), while position 2 (δ , 6.53 ppm) is less, and position 1 (δ , 7.67 ppm) is the least active site for electrophilic attack.

It has to be mentioned that the ¹H NMR spectrum also has two weak signals that can be attributed to fluorination in position 1. A signal at δ 7.17 ppm can be assigned to proton H₂: it is a doublet becoming a singlet after fluorine decoupling; it also has the same coupling constant value, $J = 10.8$ Hz as was found for H₁ coupled with the adjacent fluorine atom in 2-MFRA. An additional weak singlet at 6.78 ppm could belong to the proton H₄; other signals (H₅, H₇, and H₈) should overlap with corresponding protons from other isomers. The ¹⁹F NMR has a very weak signal with $\delta = -131.88$ ppm, which corresponds to a fluorine atom in *meta*-position to the phenol group [7] and also can be attributed to 1-MFRA. This isomer represents only a minor fraction of the total mixture of the monofluorinated resazurin.

Difluororesazurin also contained a mixture of isomers, which were separable as two different fractions by the Synergy Hydro-RP column. Based on the proton NMR spectrum we found that one fraction consists of 2,5-DFRA, and the total yield of this product is 0.8%. Addition of the second fluorine atom caused a downfield shift of the proton signals compared with the parent compound and corresponding monofluorinated derivatives. The two protons in *ortho*-positions to the hydroxyl group undergo further downfield shift: the H₄ from δ 6.13 ppm (in RA) and 6.47 ppm in 2-MFRA becomes 6.71 ppm; the H₇ from δ 6.53 ppm (RA) and 6.86 ppm (both 2-MFRA and 4-MFRA) becomes 6.99 ppm. Protons in *meta*-positions to the hydroxyl group undergo an opposite upfield shift by 0.14 ppm in

comparison with correspondent monofluorinated derivatives: H₁ has δ 7.61 ppm vs 7.74 ppm and H₈ has δ 7.97 ppm vs 8.11 ppm in 2-MFRA. ¹⁹F NMR shows the presence of a signal with δ -162.66, which can be attributed to fluorine in both the 2- and 5-positions.

The second difluororesazurin HPLC fraction is a mixture of two isomers, containing about 2/3 4,5-DFRA (total yield 1%) and 1/3 2,4-DFRA (yield 0.5%). The proton NMR spectrum of symmetrical 4,5-DFRA contains only two signals at δ 6.91 ppm in the *ortho*- (a doublet of doublets, which turns into a doublet when fluorine is decoupled) and a doublet at 7.99 ppm from the *meta*-position to the hydroxyl group. For 2,4-DFRA the positions of the protons in the non-fluorinated ring are close to corresponding signals of the parent compound (δ 6.22 ppm vs 6.13 ppm for H₅; 6.50 ppm vs 6.53 ppm for H₇; 7.71 ppm vs 7.67 ppm for H₈); the position of proton H₁ in the fluorinated ring is shifted downfield (δ 7.47 ppm). ¹⁹F NMR shows the presence of two signals: a more intense signal at δ -163.58 ppm and -162.59 ppm. We presume that the first signal can be attributed to fluorine atoms in separate rings in 4,5-DFRA, while the second one belongs to atoms located in the same ring in 2,4-DFRA.

The trifluorinated product is represented by only one isomer 2,4,5-TFRA, which appears as a separate fraction on HPLC. The positions of the proton signals on NMR (δ 6.97 ppm for H₇, 7.86 ppm for H₁, and 7.99 ppm for H₈) are close to the values for difluorinated derivatives. Two signals (δ 6.97 ppm and 7.86 ppm) have coupling with fluorine atoms, which is removed in the decoupling experiment. ¹⁹F NMR shows presence of two signals with δ -161.02 and -162.84 ppm, which belong to the signals from different aromatic rings.

Resazurin and its fluorinated compounds act like pH indicators. The literature data on the pK_a of resazurin are quite inconsistent. The pK_a value of 6.71 [8] is definitely above the pH range of indicator color change from 3.8 to 6.5 presented in the same source. To clarify this issue, we determined the pK_a value of resazurin and its fluorinated derivatives by measuring the dependence of the absorption of the basic form on the pH of solution (Figure 2). The pK_a values of resazurin and its derivatives are presented in Table 1. Our results show that the pK_a value of resazurin is 5.59, which is considerably lower than the literature value of 6.71. Introduction of each fluorine atom into the molecule of resazurin causes a decrease in the pK_a value by 0.5–0.75, similar to what was observed in triarylmethane-based pH indicators [2–4].

The absorption maxima of in the visible light range are also affected by introduction of fluorine atoms. Under acidic conditions resazurin has a weak absorption in the 500–550 nm range with no sharp maximum. Under basic conditions, it has a strong absorption at 598 nm. The introduction of fluorine atoms causes bathochromic shift of this maximum to 606 nm for monofluorinated and 619 nm for trifluorinated derivatives, which is consistent with the behavior of the triphenylmethanes (bathochromic shift by 3–7 nm per fluorine atom [2–4]). Absorption maxima of difluorinated derivatives do not follow this trend, having a slightly lower than expected value of 600 nm for 2,5-DFRA and a higher value of 622 nm for the mixture of 4,5-DFRA and 2,4-DFRA. A summary of the spectroscopic data and the acid-base properties of the fluorinated derivatives of resazurin and its analogs is presented in Table 1.

Resazurin is reduced by metabolically active cells into resorufin, which is usually observed by fluorescence spectroscopy. In order to determine the possibility of application of the fluorinated derivatives of resorufin, we performed fluorination of resorufin using the same method. The acidic form of resorufin is not soluble in acetic acid (as well as in water and other organic solvents), but readily dissolves in trifluoroacetic acid giving an orange solution, most likely arising from the protonated form of the molecule. Bubbling of 0.1% fluorine gas through the solution of resorufin in trifluoroacetic acid resulted in one major product at about 10% yield. Mass-spectrometry and ^1H NMR analysis have shown that it is represented as a mixture of 4-monofluoresorufin (4-MFRR; overall yield 6%) and of 2-monofluoresorufin (2-MFRR; overall yield 4%), as it is shown on Figure 3. We also observed a very small amount (yield 0.3–0.5%) of another product, which was characterized by mass-spectrometry as difluoresorufin (DFRR). We were not able to collect significant amount of DFRR to perform further characterization by NMR. Still we were able to analyze acid-base and optical properties of both fluorinated products. We determined the following K_a (and pK_a) values: $1.6 \cdot 10^{-6}$ M (5.80) for resorufin, $6.0 \cdot 10^{-6}$ M (5.22) for MFRR, and $4.4 \cdot 10^{-5}$ M (4.36) for DFRR. The basic forms of all the products were found to be highly fluorescent with excitation/emission wavelengths 568/585 nm for resorufin, 576/594 nm for MFRR, and 588/601 nm for DFRR. These data show that the introduction of fluorine atoms caused the same effects (decreasing of pK_a and bathochromic shift of absorption), as was observed for triarylmethane indicators [2–4] and for the resazurin derivatives.

Summary of experimental results

Resazurin— m/z^+ 230.046, $pK_a = 5.59$, $\lambda_{\text{basic}} = 598$ nm,

^1H NMR (360 MHz, D_2O) δ : 6.13 (s, 2H: H_4 , H_5); 6.53 (d, $J = 9.7$ Hz, 2H: H_2 , H_7); 7.67 (d, $J = 9.7$ Hz, 2H: H_1 , H_8).

Monofluoresazurin— m/z^+ 248.036; for mixture of 4/5 4-MFRA and 1/5 2-MFRA: $pK_a = 4.88$, $\lambda_{\text{basic}} = 606$ nm

2-MFRA: ^1H NMR (360 MHz, D_2O , δ): 6.44 (d, $J_{\text{HF}} = 9.7$ Hz, 1H: H_4); 6.61 (s, 1H: H_5); 6.86 (d, $J = 9.7$ Hz, 1H: H_7); 7.74 (d, $J_{\text{HF}} = 10.8$ Hz, 1H: H_1); 8.11 (d, $J = 9.7$ Hz, 1H: H_8).

^1H { ^{19}F } NMR removes coupling of signals at δ 6.44 and 7.74

^{19}F NMR (338 MHz, D_2O): δ -163.64

4-MFRA: ^1H NMR (360 MHz, D_2O , δ): 6.61 (s, 1H: H_5); 6.86 (d, $J = 9.7$ Hz, 1H: H_7); 6.89 (dd, $J_{\text{HF}} = 7.9$, $J_{\text{HH}} = 9.7$ Hz, 1H: H_2); 7.95 (d, $J = 9.7$ Hz, 1H: H_1); 8.11 (d, $J = 9.7$ Hz, 1H: H_8).

^1H { ^{19}F } NMR converts 6.89 into d, $J = 9.7$ Hz.

^{19}F NMR (338 MHz, D_2O): δ -163.64

Difluoresazurin— m/z^+ 266.026, for mixture of 2/3 4,5-DFRA and 1/3 2,5-MFRA: $pK_a = 4.08$, $\lambda_{\text{basic}} = 622$ nm;

4,5-DFRA: ^1H NMR (360 MHz, D_2O , δ): 6.91 (*dd*, $J_{\text{HH}} = 9.7$ Hz, $J_{\text{HF}} = 8.6$ Hz, 2H: H_2 , H_7); 7.99 (*d*, $J = 9.7$ Hz, 2H: H_1 , H_8).

^1H { ^{19}F } NMR converts 6.91 into *d*, $J = 9.7$ Hz.

^{19}F NMR (338 MHz, D_2O): δ -163.58

2,4-DFRA: ^1H NMR (360 MHz, D_2O , δ): 6.22 (*s*, 1H: H_5); 6.50 (*d*, $J = 8.3$ Hz, 1H: H_7); 7.47 (*d*, $J_{\text{HF}} = 9.7$ Hz 1H: H_1); 7.71 (*d*, $J_{\text{HF}} = 8.3$ Hz, 1H: H_8).

^1H { ^{19}F } NMR converts 7.47 into *s*.

^{19}F NMR (338 MHz, D_2O): δ -162.59.

2,5-DFRA: m/z^+ 266.026, $pK_a = 4.16$, $\lambda_{\text{basic}} = 600$ nm;

^1H NMR (360 MHz, D_2O , δ): 6.71 (*d*, $J_{\text{HF}} = 7.2$ Hz, 1H: H_4); 6.99 (*dd*, $J_{\text{HH}} = 9.7$ Hz, $J_{\text{HF}} = 8.6$ Hz 1H: H_7); 7.61 (*d*, $J_{\text{HF}} = 10.8$ Hz, 1H: H_1); 7.97 (*d*, $J = 9.7$ Hz: H_8).

^1H { ^{19}F } NMR converts 6.71 into *s*; 6.99 into *d*, $J = 9.7$ Hz; and 7.61 into *s*.

^{19}F NMR (338 MHz, D_2O): δ -162.66

2,4,5-Trifluororesazurin (2,4,5-TFRA): m/z^+ 286.018, $pK_a = 3.59$, $\lambda_{\text{basic}} = 619$ nm.

^1H NMR (360 MHz, CD_3OD , δ TMS): 6.97 (*dd*, $J_{\text{HH}} = 9.4$ Hz, $J_{\text{HF}} = 10.8$ Hz 1H: H_7); 7.86 (*d*, $J_{\text{HF}} = 10.8$ Hz, 1H: H_1); 7.99 (*d*, $J = 9.4$ Hz, 1H: H_8).

^1H { ^{19}F } NMR converts 6.97 into *d*, $J = 9.4$ Hz and 7.86 into *s*.

^{19}F NMR (338 MHz, CD_3OD): δ -161.02, -162.84

Resorufin— m/z^+ 214.052, $pK_a = 5.6$, $\lambda_{\text{excitation}} = 568$ nm, $\lambda_{\text{emission}} = 585$ nm.

^1H NMR (360 MHz, D_2O , δ): 6.13 (*d*, $J = 2.2$ Hz, 2H: H_4 , H_5); 6.53 (*dd*, $J = 9.3$, 2.2 Hz, 2H: H_2 , H_7); 7.10 (*d*, $J = 9.3$ Hz, 2H: H_1 , H_8).

Monofluororesorufin— m/z^+ 232.043, for mixture of 3/5 of 4-MFRA and 2/5 of 2-MFRA $pK_a = 5.1$, $\lambda_{\text{excitation}} = 576$ nm, $\lambda_{\text{emission}} = 594$ nm.

2-MFRR: ^1H NMR (360 MHz, D_2O , δ) 6.50 (*d*, $J = 2.2$ Hz, 1H: H_5); 6.55 (*d*, $J = 2.2$ Hz, 1H: H_4); 6.84 (*dd*, $J = 9.4$, 2.2 Hz, 1H: H_7); 6.86 (*d*, $J_{\text{HF}} = 9.4$ Hz, 1H: H_1); 7.60 (*d*, $J = 9.4$ Hz, 1H: H_8).

^1H { ^{19}F } NMR converts 6.86 into *s*.

^{19}F NMR (338 MHz, D_2O): δ -164.80 (*d*, $J_{\text{HF}} = 9.4$ Hz, 1F)

4-MFRR: ^1H NMR (360 MHz, D_2O , δ): 6.50 (*d*, $J = 2.2$ Hz, 1H: H_5); 6.84 (*dd*, $J = 9.4$, 2.2 Hz, 1H: H_7); 6.90 (*d*, $J = 9.4$ Hz, 1H: H_2); 7.50 (*d*, $J = 9.4$ Hz, 1H: H_1); 7.60 (*d*, $J = 9.4$ Hz, 1H: H_8).

^{19}F NMR (338 MHz, D_2O): δ -164.81 (*s*, 1F)

Difluororesorufin— m/z^+ 250.033, $pK_a = 4.6$, $\lambda_{\text{excitation}} = 588 \text{ nm}$, $\lambda_{\text{emission}} = 601 \text{ nm}$.

Discussion

Resazurin represents another group of complex organic molecules, which can be selectively fluorinated by diluted fluorine gas in acidic conditions with introduction of an ^{18}F -label into the molecule. Although the yield of the products in this reaction (1–2.5%) is much lower in comparison with fluorine addition to double bonds (30%) [9] and fluorination of triarylmethane pH indicators (5–10%) [2–4], it is still high enough for preparation of the ^{18}F -labeled compound at millicurie level, which is enough for practical application in any biological system.

Introduction of the first fluorine atom into the molecule of resazurin occurs at *ortho* positions to the hydroxyl group. This results in substitution of the most active hydrogen atom in position 4 and (to a lesser extent) in position 2 (Fig. 1). Addition of the next fluorine atom could be complicated by the fact that the aromatic system now contains two *ortho-para* orienting groups in adjacent positions, where their effect is expected to be opposite. However, addition of the second and the third fluorine atoms occurs exclusively into positions *ortho* to the hydroxyl group. This result suggests that the contribution of the OH group for orientation of electrophilic substituents in aromatic systems is dominant over the effect of the fluorine atom. The main products of multiple fluorination of resazurin are 4,5-, 2,5-, and 2,4- difluororesazurin, and 2,4,5-trifluororesazurin. We were not able to detect two other potential products of fluorination into the *ortho*-positions: 2,7- difluororesazurin and 2,4,7-trifluororesazurin. These were most likely produced in very small quantities because of the preferable initial fluorine attack into position 4/5 rather than 2/7.

^{18}F -labeled light-absorbing and fluorescent molecules represent a new type of probe for *in vivo* studies of biological systems. The decay of the radioactive label results in emission of high energy positrons, which produce two types of electromagnetic radiation. Because the velocity of these particles exceeds the speed of light in water, they initially emit Cerenkov luminescence in the UV-visible range of the spectrum. Upon contact with an electron, they annihilate producing two oppositely directed 511 keV gamma photons. By combining light absorbing compounds with the positron emitter within the same molecule, we create probes with a unique combination of properties: they provide functional information as the visible light interacts with its selective quencher, with the gamma-radiation providing for quantitation of probe amount and localization of the target.

Previously, we developed ^{18}F -labeled pH indicators [2–4] and used them for *in vivo* assessment of the hydrogen ion concentration by selective quenching of the Cerenkov luminescence under basic conditions [6]. In this paper, we present ^{18}F -labeled fluorinated derivatives of resazurin, which have both light absorption and fluorescence. The molecules of the unmodified probe (resazurin) absorb the light, causing a decrease of the intensity of Cerenkov luminescence. After *in vivo* reduction by viable cells they are converted into fluorescent molecules, which cause increasing of the light intensity due to secondary Cerenkov-induced fluorescence [10, 11]. As the result, an overall intensity of the observed light will be increased proportionally to the concentration of viable cells in the tissue of

target (e.g. tumor). The presence of the radioactive label will also allow quantification of the probe amount and its biodistribution by PET, providing precision and reliability to the measurement.

The practical application of this kind of probe requires a specific set of optical properties. The parent compound resazurin is widely used in cell culture as a cell viability assay and for detection of cell presence *in vitro* (e.g. bacteria in milk) [6]. However, its *in vivo* application presents some challenges: it is reduced by viable cells into resorufin, which fluoresces with excitation at 568 nm and emission at 585 nm. Both excitation and emission maxima of resorufin are very close to the absorption maximum of oxyhemoglobin (578 nm) [12], and therefore the intensity of the fluorescence in biological objects will be affected by tissue oxygenation status. Introduction of the fluorine atoms into the probe molecule causes a bathochromic shift of the absorption and fluorescence maxima and has the potential to eliminate this obstacle. The excitation/emission wavelengths of monofluororesorufin (576/594 nm) are still not optimal, but for difluororesorufin they have higher values (588/601 nm), which should start to diminish an influence of hemoglobin and other porphyrin-based molecules on our ability to detect the probe. We were not able to synthesize and characterize trifluororesorufin, but based on the trend for other derivatives it should have even higher values of excitation and emission maxima (estimated numbers are 595/610 nm), making di- and trifluororesazurin the most promising probes for *in vivo* observation of the cell viability.

The acid-base properties of the probes also need to be taken into account for their practical application. The probe reduction occurs as an intracellular NADPH-driven process, which requires a transport of the molecules into the cells. Resazurin readily enters the cells, and the same ability will supposedly be shared by its fluorinated derivatives. However, this process can be altered by the charge of the molecules. Neutral molecules are known to penetrate cellular membranes much better than correspondent negatively charged anions [13]. The literature value for the pK_a of resazurin is 6.71, suggesting that at normal extracellular pH 7.4 about 20% of the molecules will be in the uncharged acidic form, while the rest is an anion. If intracellular transport occurs for the neutral form only, it still could provide enough probe concentration to observe its reduction. Introduction of the fluorine atom into the molecules makes them more acidic [2–4], suggesting a significant decrease of the fraction of the neutral non-dissociated molecules at biologically significant pH. This alteration of the charge could affect intracellular transport of the molecules and limit the application of the probe. Therefore we performed an analysis of acid-base behavior of resazurin (Fig. 2) and found that its pK_a had a much lower value of 5.59. Because of this an overwhelming majority of its molecules (98.5%) will be negatively charged at biological pH 7.4, suggesting insignificance of the charge for intracellular transport of resazurin. Therefore the more acidic fluorinated derivatives of resazurin should also have no limitation on their intramolecular transport and will be able to function as *in vivo* cell viability probes.

Conclusions

Direct fluorination of resazurin and resorufin with [^{18}F]- F_2 in acidic conditions leads to the synthesis of ^{18}F -labeled derivatives. These compounds have an optimal combination of the

optical and acid-base properties for *in vivo* investigation of cell viability in biological objects using combination of PET and optical Cerenkov imaging.

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Figure 1.

Direct fluorination reaction of resazurin in acetic acid by 0.1% F_2 – Ne gas mixture. The fluorine atoms are introduced into the *ortho*-position relative to the phenolic hydroxyl group, resulting in 4- and 2-monofluororesazurin (ratio 4:1). The second fluorine atoms also substitute hydrogen atoms in *ortho*-positions, producing 4,5-, 2,5-, and 2,4-difluororesazurin (ratio 4:3:2). Further fluorination leads to 2,4,5-trifluororesazurin. The percent numbers indicate the actual yields of the correspondent isomers.

Figure 2.

Titration curve of resazurin (1) shows that its pK_a value is equal to 5.59, which is lower than the current literature data (6.71 [8]). Addition of fluorine atoms into the resazurin molecule makes it more acidic, decreasing the pK_a values for monofluororesazurins (curve 2, mixture of 80% of 4-MFRA and 20% of 2-MFRA), difluororesazurins (curve 3 for 2,5-DFRA and curve 4 for 67% 4,5-DFRA and 33% 2,4-DFRA), and trifluororesazurin (curve 5 for 2,4,5-TFRA).

Figure 3.

Reaction of resorufin with 0.1% F_2 – Ne gas mixture in trifluoroacetic acid results in fluorination of the *ortho*-position to the phenolic hydroxyl group with production of 4- and 2-monofluoresazurin.

Table 1

Acid-base and optical properties of fluorinated derivatives of resazurin.

Compound	Basic form λ_{max} , nm	K_a	pK_a
Resazurin	598	$2.6 \cdot 10^{-6}$	5.59
4-MFRA + 2-MFRA	606	$1.3 \cdot 10^{-5}$	4.88
2,5-DFRA	600	$6.9 \cdot 10^{-5}$	4.16
4,5-DFRA+2,4-DFRA	622	$8.1 \cdot 10^{-5}$	4.09
2,4,5-TFRA	619	$2.5 \cdot 10^{-4}$	3.59