Reactivity of paraquat with sodium salicylate: Formation of stable complexes

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Abstract

Sodium salicylate (NaSAL) has been shown to be a promising antidote for the treatment of paraquat (PQ) poisonings. The modulation of the pro-oxidant and pro-inflammatory pathways, as well as the anti-thrombogenic properties of NaSAL are probably essential features for the healing effects provided by this drug. Nevertheless, a possible direct chemical reactivity between PQ and NaSAL is also a putative pathway to be considered, this hypothesis being the ground of the present study. In accordance, it is shown, for the first time that PQ and NaSAL react immediately in aqueous medium and within 2–3 min in the solid state. Photographs and scanning electron photomicrographs indicated that a new chemical entity is formed when both compounds are mixed. This assumption was corroborated by the evaluation of the melting point, and through several analytical techniques, namely ultraviolet/visible spectroscopy, nuclear magnetic resonance spectroscopy, gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS), liquid chromatography/electrospray ionization/mass spectrometry/mass spectrometry (LC/ESI/MS/MS) and infrared spectroscopy, which revealed that stable charge-transfer complexes are formed when PQ is mixed with NaSAL. LC/ESI/MS/MS allowed obtaining the stoichiometry of the charge-transfer complexes. In order to increase resolution, single value decomposition, acting as a filter, showed that the charge-transfer complexes with m/z 483, 643 and 803 correspond to the pseudo-molecular ions, respectively 1:2, 1:3 and 1:4 (PQ:NaSAL). In conclusion, these results provided a new and important mechanism of action of NaSAL against the toxicity mediated by PQ.

Keywords:
Paraquat
Sodium salicylate
Charge-transfer complexes

Introduction

Salicylate and its derivatives represent, perhaps, one of the most interesting pharmacologic compounds with many undisclosed therapeutic properties (Brunton et al., 2006). Recently, our research group showed an additional application for this drug, by demonstrating that sodium salicylate (NaSAL) could represent a powerful antidote to be used against paraquat (PQ) poisonings (Dinis-Oliveira et al., 2007a). In that study, the remarkable healing effects obtained by the administration of NaSAL, asserted by histological, functional, and survival analysis, were shown to be associated to an effective inhibition of pro-inflammatory factors such as NF-κB, to the scavenging of reactive oxygen species (ROS), and also through the inhibition of myeloperoxidase activity and inhibition of platelet aggregation. A subsequent study reinforced the potential use of this interesting molecule in the protection

Abbreviations: CT, charge-transfer; ESI, electrospray ionization; GC/MS/MS, gas chromatography/mass spectrometry/mass spectrometry; IR, infrared; LC/ESI/MS/MS, liquid chromatography/electrospray ionization/mass spectrometry/mass spectrometry; m.p., melting point; NaSAL, sodium salicylate; NF-κB, nuclear factor kappa-B; NMR, nuclear magnetic resonance; PCA, principal component analysis; PQ, paraquat; ROS, reactive oxygen species; SEM, scanning electron microscopy; SVD, singular value decomposition; UV/vis, ultraviolet/visible.

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against PQ-induced lung apoptosis (Dinis-Oliveira et al., 2007b). The obtained results were fully satisfactory since not only the toxicity was reverted but also, most significantly, the full survival of the PQ-intoxicated rats treated with NaSAL (extended for more than 30 days) in contrast to 100% of mortality by the day 6 in PQ-only exposed animals (Dinis-Oliveira et al., 2007a) was observed.

Though the modulation of the pro-oxidant and pro-inflammatory pathways, as well as the anti-thrombogenic properties of NaSAL are probably essential features for the healing effects provided by this drug (Dinis-Oliveira et al., 2008, 2007a,b), a possible direct chemical reactivity between PQ and NaSAL is also a putative pathway to be considered. Corroborating this idea, we noticed that when a PQ aqueous solution is mixed with a NaSAL aqueous solution, an intensive yellow colour develops immediately in a non-reversible form. After these preliminary findings, a thorough review of PQ literature (Dinis-Oliveira et al., 2008) allowed us to find some studies from the late 1960s, indicating that many phenols form soluble crystalline charge-transfer (CT) complexes with PQ dichloride (Akhavein and Linscott, 1968; Ledwith and Woods, 1970; White, 1969). The fast formation of CT complexes was considered to be, possibly, one of the factors that change the herbicidal activity of PQ among floral species and through the plant lifespan. Significantly, the mobility (from the leaves) of PQ was lower in plants having high tannin (polyphenol) content in the leaf structure (Ledwith and Woods, 1970; White, 1969). Furthermore, authors suggested that the formation of CT complexes between PQ (electron-deficient moiety) and the polyphenolic humic acids (electron-rich centers) may contribute to deactivation of the herbicide in the soils. More recently, Pacheco et al. (2003) and Gevao et al. (2000) corroborated these results and showed charge neutralization between the several negatively charged groups in humic acids and the positively charged PQ. Thus, it must be noted that the establishment of CT complexes between polyphenolic compounds and bipyridylium herbicides such as PQ may be important in controlling the absorption, mobility and mode of action of the herbicide in plants. Our hypothesis is that NaSAL, may form CT complexes with PQ, exhibiting, therefore, a chemical behaviour close to that of polyphenols. Thus, the aim of the present study was to evaluate the chemical reactivity of PQ with NaSAL.

Materials and methods

Chemicals and drugs

Paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride; molecular mass = 257.2 g/mol), NaSAL (2-hydroxybenzoic acid sodium salt; molecular mass = 160.11 g/mol), acetone, chloroform, acetonitrile, diethyl ether, *n*-hexane, benzene, sodium benzoate, phenol, acetylsalicylic acid and lysine acetylsalicylate were all obtained from Sigma (St. Louis, MO, USA). All solvents were of analytical or HPLC grade.

Role of the hydroxyl group

The first approach was to assess the reactivity of the different functional groups of NaSAL with PQ. Therefore, benzene, sodium benzoate, phenol, acetylsalicylic acid and lysine acetylsalicylate were mixed with PQ following the stoichiometry of 1:1, 1:2, 1:3 and 1:4 (PQ:evaluated compound). The appearance (or not) of an intensive yellow colour was registered.

Preparation of crystalline charge-transfer complexes between paraquat with sodium salicylate

CT complexes were obtained following a previously described procedure for polyphenols, with slight modifications (Ledwith and Woods, 1970). PQ and NaSAL [0.2 g:3.2 g (1:10)] were warmed with carbon tetrachloride (10 mL) to produce a yellow-orange solution. Addition of ether (2 mL) resulted in the formation of bright yellow crystals. The ethereal phase (containing crystals) was separated, followed by evaporation of ether to dryness in vacuum in order to obtain crystals. Further yield of crystals were obtained after cooling and careful addition of more ether.

Scanninge lectron microscopy

PQ, NaSAL and CT complexes were air-dried, mounted on a stub, sputter coated with gold/palladium, and examined with a JSM scanning electron microscope (model 5400) at 10 kV. The magnifications used are depicted in Fig. 3.

Melting points and solubilities of paraquat, sodium salicylate and of the charge-transfer complexes

The melting point (m.p.) was measured on a Reichert-Thermovar hot-stage apparatus (Leica, Wetzlar, Germany) and on a Kofler apparatus. Solubilities (1 g/L) were recorded at 20 °C for water, methanol, *n*-hexane, ethyl acetate, dichloromethane, chloroform, acetone and acetonitrile.

Ultraviolet/visible spectroscopy

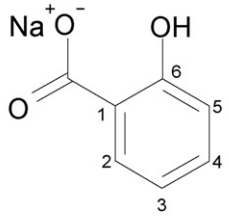
The ultraviolet/visible (UV/vis) spectra were recorded in water using a path length of 1 cm, at room temperature on a plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA). Qualitative analysis of spectra was made in the wavelengths range of 200–340 and 200–600 nm, satisfying or not satisfying the Lambert–Beer law, respectively.

Infrared spectroscopy

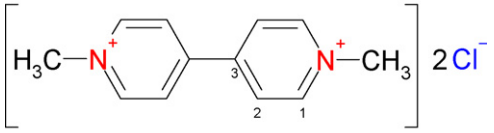
IR spectra were obtained using a Mattson ATI Genesis FT-IR spectrometer. Samples let at 37 °C for 20 min were gently mixed with 300 mg of micronized KBr powder and compressed into discs at a force of 10 kN for 2 min using a manual tablet presser (PerkinElmer, Norwalk, USA). All the samples were examined as KBr disks. The spectra were smoothed with a factor of 4 on a scale of 1–16 (1 the least and 16 the maximum of smoothing). No purging was carried out. IR spectra were recorded in the region of 500–4000 cm^{-1}. Win FIRST software was used to control the spectrometer and to acquire and manipulate spectra.

Nuclear magnetic resonance spectroscopy

^{1}H and ^{13}C NMR spectra were recorded in deuteriochloroform on a Bruker Avance 300 spectrometer (Wissembourg, France) operating at 300.13 and 75.47 MHz, respectively. Chemical shifts are expressed in δ(ppm) values relative to tetramethylsilane ($\delta = 0$) as external reference. Unequivocal ^{13}C assignments were made on the basis of 2D (^{1}H,^{13}C) HSQC and HMBC (delay for long-range *J* C/H couplings were optimized for 7 Hz) experiments. The phase sensitive ^{1}H-detected (^{1}H,^{13}C) *g*HSQC (heteronuclear single quantum coherence, using gradient pulses for selection) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 1500 Hz in F_2 and 7000 Hz in F_1. A cosine multiplication was applied in both dimensions. The delays were adjusted according to a coupling constant ^{1}J(CH) of 149 Hz. The *g*HMBC (heteronuclear multiple quantum coherence, using gradient pulses for selection) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 1500 Hz in F_2 and 7500 Hz in F_1. A sine multiplication was applied in both dimensions. The low-pass *J*-filter of the experiment was adjusted for an average coupling constant ^{1}J(CH) of 149 Hz and the long-range delay utilized to excite the heteronuclear multiple quantum coherence was optimized for 7 Hz.



NaSAL (2-hydroxybenzoic acid sodium salt)



PQ (1,1′-dimethyl-4,4′-bipyridylium dichloride)

Fig. 1. Chemical structures of paraquat (PQ) and sodium salicylate (NaSAL).

Gas chromatography/mass spectrometry/mass spectrometry

PQ, NaSAL and the CT complexes in methanol were analyzed using a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8. The chromatographic column used was a VF-5 ms (30 m × 0.25 mm × 0.25 μm) from VARIAN. The injector port was heated to 220 °C. The split vent was opened after 30 s and the ratio split was 1/40. The carrier gas was helium (Gasin, Portugal), at 1 mL/min, constant flow. The oven temperature was 40 °C (for 1 min), then increased at 2 °C/min to 220 °C and held for 30 min. All mass spectra were acquired in the electron impact (EI) mode. Ionization was maintained off during the first 4 min, to avoid solvent overloading. The Ion Trap detector was set as follows: the transfer line, manifold and trap temperatures were 280, 50 and 180 °C, respectively. The mass range was 50–800 m/z, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25,000 μs, with an ionization storage level of 35 m/z. The injection volume was 1 μL and the analysis was performed in FullScan mode.

Liquid chromatography/mass spectrometry/mass spectrometry

LC analysis was carried out on a Prostar 210 LC pump (Varian, CA, USA), using methanol as solvent, at a flow rate of 0.05 mL/min. A Varian 1200 triple quadrupole mass spectrometer was used with electrospray ionization (ESI). Full scan spectra were collected in the mass range of 100–1200 m/z both in positive and negative modes. The operating parameters of the ESI source were all optimized regarding the maximum signal intensity as follows: the nebulizing gas pressure was 40 psi; the drying gas pressure was 20 psi; the drying gas temperature was 200 °C; the housing temperature was 55 °C; the needle voltage was −4.3 kV at negative ion mode and 5.5 kV at positive ion mode; the shield voltage was −550 V at negative ion mode and 400 V at positive ion mode. Samples were injected by "direct infusion" using a 1 mL loop. The capillary voltage was scanned starting from 1 to 100 V with successive increments of 10 V in negative and positive mode simultaneously, originating 20 mass spectra calculated as the average of 20 scans each.

Statistical analysis of the liquid chromatography/mass spectrometry/mass spectrometry results

Singular value decomposition (SVD) is a blind signal technique widely used in multivariate signal processing. When mean centering or auto-scaling the signal, the SVD of this data performs a well known statistical analysis, the principal component analysis (PCA). Using this noise suppression strategy, the data matrix (x) of PQ, NaSAL and charge-transfer complexes obtained from the "mass spectra-fingerprint", by scanning capillary voltages in positive mode, were decomposed in order of magnitude of variation directions in the variable space (m/z). Generally, most variability is obtained in the first principal components (PC), while as in good signal to noise spectral data, noise is obtained in the last orthogonal decompositions.

Fig. 2. Photographs of the time-course reactivity of paraquat (PQ) and sodium salicylate (NaSAL), 1 min (A), 10 min (B) and 20 min (C) after mixing both powders. Photographs of PQ (D), NaSAL (E) and of the charge-transfer (CT) complexes (F) recovered as solids accordingly the method described by Ledwith and Woods (1970).

The scores projection of the samples in the new coordinate system provides "resolution" and the respective loadings calculated in SVD as the "filtered" mass spectra. They can be understood as the weights for each original variable (m/z) when calculating the principal component.

Results

Reactivity of functional groups

Chemical structures of PQ and NaSAL are showed in Fig. 1. PQ or NaSAL when dissolved in water did not result in any colour modification. However, an intensive yellow colour developed immediately when PQ and NaSAL were mixed following a stoichiometry of 1:1, 1:2 and 1:4. Concerning the benzene, sodium benzoate, phenol, acetylsalicylic acid and lysine acetylsalicylate, only the later and the phenol revealed consistent results with those obtained for NaSAL. This indicates that the hydroxyl group is involved in the development of the yellow colour.

Preparation of crystalline charge-transfer complexes of paraquat with sodium salicylate

It was observed that the reaction of PQ and NaSAL also occurred in the solid state, just by placing the powders in physical contact. In Fig. 2A–C it is shown the crystals formation. Subsequently, more pure CT complexes were obtained (Fig. 2F) following the previously described method of Ledwith and Woods (1970) with slight modifications. PQ and NaSAL photographs are shown in Fig. 2D and E, respectively.

Scanning electron microscopy

The morphology information was obtained from SEM. Fig. 3 provides SEM images of PQ, NaSAL and CT complexes crystals prepared as described above. Photomicrographs show that NaSAL powders are of polygonal and sharp shape of various sizes and with large surface areas. PQ crystals are small, shapeless and the size is not homogenous. CT complexes morphology revealed microparticles of various sizes but with round and smooth surfaces.

Melting points and solubilities of paraquat, sodium salicylate and of the charge-transfer complexes

The m.p. measured on a Reichert-Thermovar hot-stage and on a Kofler apparatus revealed comparable results. PQ and NaSAL both exhibit m.p. above 300 °C (with decomposition) and the CT complexes showed the following behaviour: from 80 to 110 °C—colour changes (yellow → orange → red → black), from 190 to 200 °C—becomes a unique compact dark solid and from 200 to 202 °C (total decomposition). Concerning the solubilities, PQ, NaSAL and the CT complexes were soluble in water and methanol

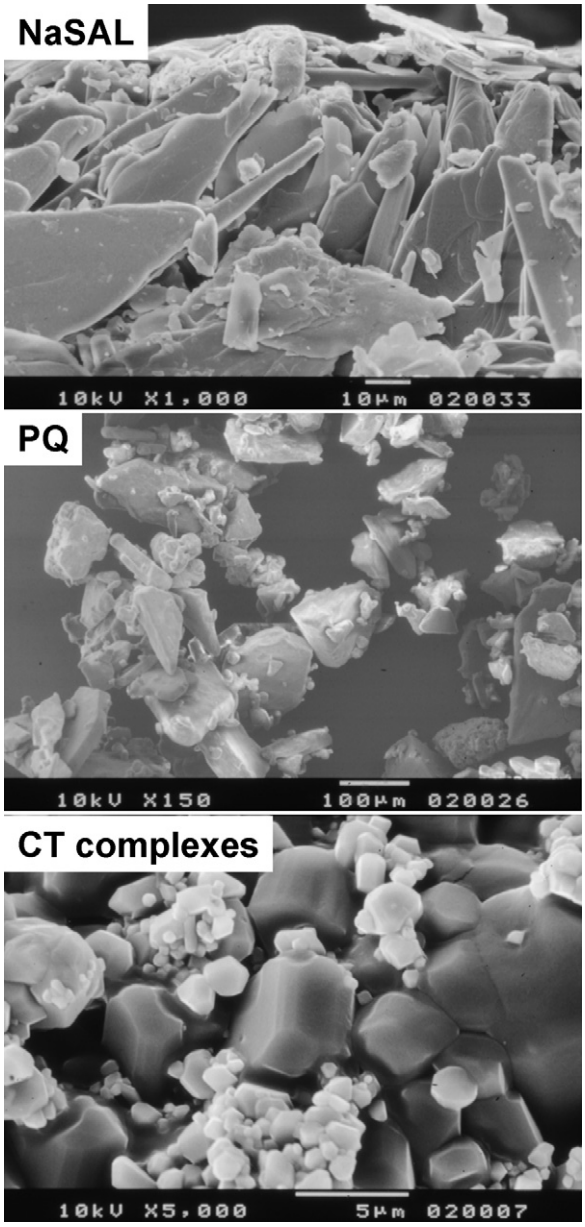


Fig. 3. Scanning electron microscopy (SEM) micrographs of sodium salicylate (NaSAL), paraquat (PQ) and of the charge-transfer (CT) complexes recovered as solids accordingly the method described by Ledwith and Woods (1970).

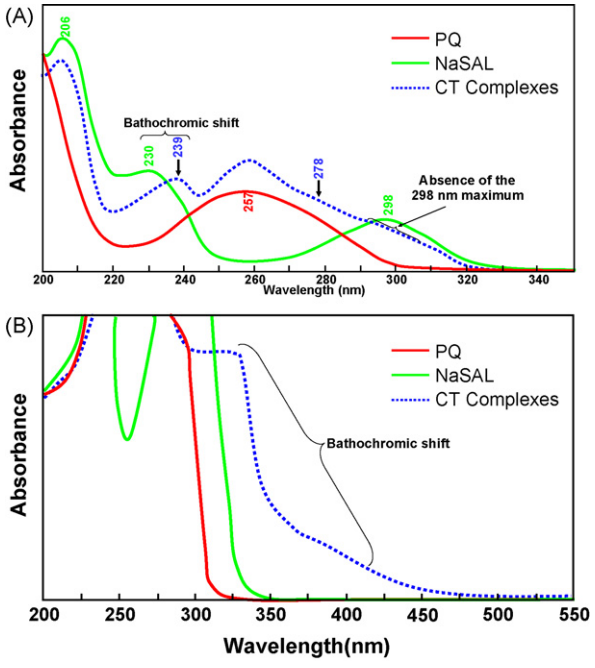


Fig. 4. Ultraviolet/visible spectra of sodium salicylate (NaSAL), paraquat (PQ) and of the charge-transfer (CT) complexes. A and B spectra acquired satisfying or not satisfying the linear range of the Lambert–Beer law, respectively.

(protic solvents) and insoluble in acetone, chloroform, acetonitrile, diethyl ether, *n*-hexane and benzene (nonprotic solvents).

UV/vis spectroscopy

The UV/vis spectra of PQ, NaSAL and of the CT complexes are depicted in Fig. 4A and B. PQ showed a single absorption maximum at 257 nm. The UV/vis spectrum of NaSAL showed three absorption maxima at 206, 230 and 298 nm. The CT complexes formed between PQ and NaSAL revealed a slightly different spectrum. The NaSAL at 230 nm maximum was shifted to 239 nm (bathochromic effect) and the absorption maximum at 298 nm disappeared. These correspond to the most significant alterations achieved satisfying the Lambert–Beer law (Fig. 4A). Outside the linear range (Fig. 4B) absorbance between 350 and 550 nm (visible region) was only possible to observe in the spectrum obtained from the CT complexes.

Infrared spectroscopy

The IR spectra of PQ, NaSAL and of the CT complexes are shown in Fig. 5. The main absorption bands of PQ were observed at 3429, 2990, 1639, 1558, 1504, 1351, 1264, 1230, 1176 and 813 cm^{-1}. The main absorption bands for NaSAL were observed at 1580, 1483, 1463, 1374, 1316, 1293, 1245, 1138, 981, 857, 807, 741 and 697 cm^{-1}. The IR spectrum obtained from CT complexes was essentially a combination of the spectra of the components except that an additional band appeared at 3121 cm^{-1} (possibly corresponding to hydrogen-bonded alcohols, aromatic C–H and alkenes C–H

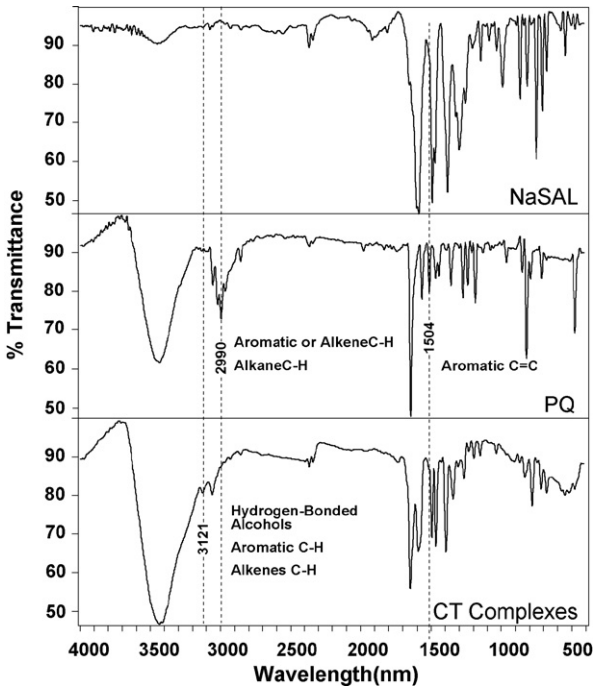


Fig. 5. Infrared spectra of sodium salicylate (NaSAL), paraquat (PQ) and of the charge-transfer (CT) complexes.

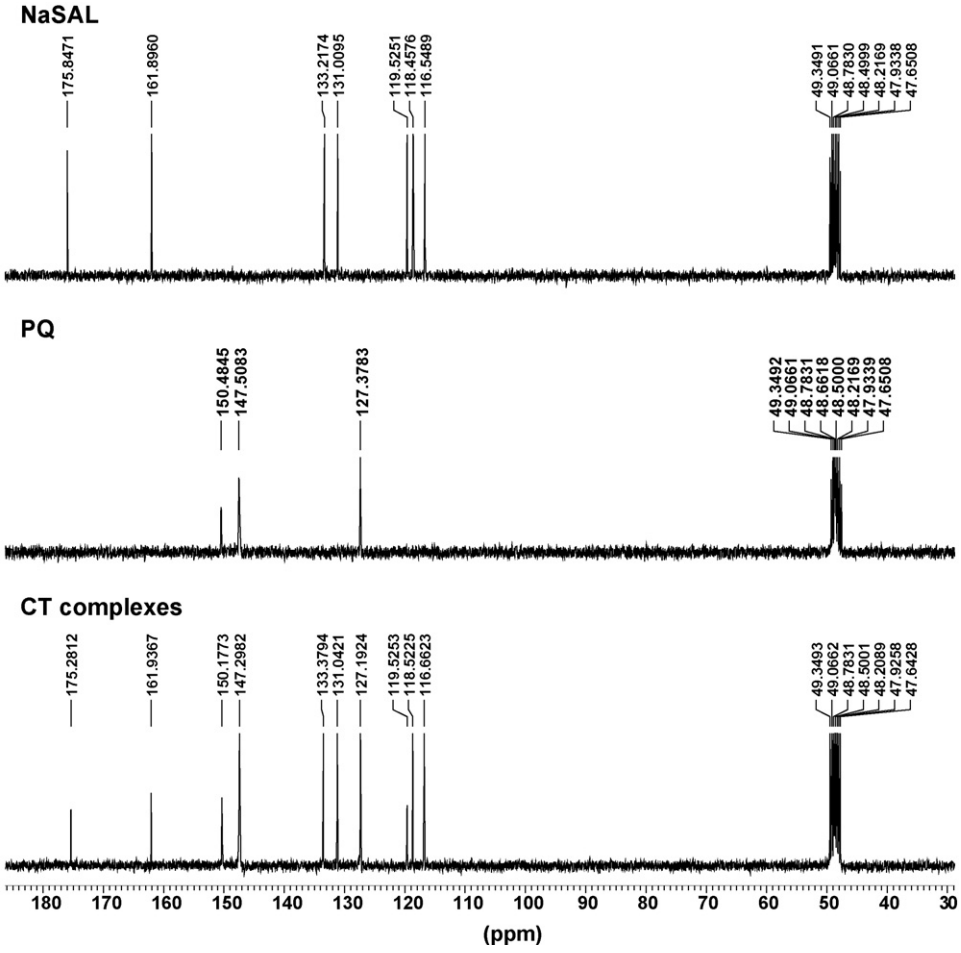


Fig. 6. ^{13}C nuclear magnetic resonance spectra obtained for sodium salicylate (NaSAL), paraquat (PQ) and of the charge-transfer (CT) complexes.

Table 1
^{1}H and ^{13}C NMR signals (ppm, in deuterochloroform) for paraquat (PQ), sodium salicylate (NaSAL) and CT complexes

Proton numbers[a]	NaSAL	PQ	CT complexes	δ
Solution ^{1}H NMR				
3S	6.82		6.71	(−) 0.11
4S	7.27		7.23	(−) 0.04
5S	6.78		6.73	(−) 0.05
6S	7.87		7.72	(−) 0.15
1P		9.21	9.12	(−) 0.09
2P		8.70	8.56	(−) 0.14
CH_3		4.55	4.50	(−) 0.05

Carbon numbers[a]	NaSAL	PQ	CT complexes	δ
Solution ^{13}C NMR				
1S	119.5		119.5	0.0
2S	161.9		161.9	0.0
3S	116.6		116.7	0.1
4S	133.2		133.4	0.2
5S	118.5		118.5	0.0
6S	131.0		131.0	0.0
CO_2H	175.8		175.3	(−) 0.5
1P		150.5	150.2	(−) 0.3
2P		127.4	127.2	(−) 0.2
3P		147.5	147.3	(−) 0.2
CH_3		48.7	48.6	(−) 0.1

Chemical shifts data (δ) obtained.
[a] For details see Fig. 1.

stretching) and absorptions at 2990 (possibly corresponding to an aromatic or alkene C–H, or an alkane C–H bond) and 1504 (corresponding to an aromatic C=C bond) were not present.

Nuclear magnetic resonance spectroscopy

^{1}H and ^{13}C NMR chemical shifts data (δ, ppm, in deuterochloroform) for PQ, NaSAL and of the CT complexes are shown in Table 1. It is important to note that in the CT complexes, the proton signals of both structures moved upfield, probably due to the anisotropic shielding effects of the aromatic rings of both structures spatially close. The charge transfer from NaSAL (electron-rich aromatic rings) to PQ (electron-poor pyridinium rings) seems to be more evident from the ^{13}C NMR of the CT complexes (Table 1 and Fig. 6), since all the carbon resonances of NaSAL are shielded and some ones from NaSAL are deshielded.

Gas chromatography-mass spectrometry – mass spectrometry

The GC/MS chromatogram of the CT complexes in methanol is shown in Fig. 7A. The peaks corresponding to PQ and NaSAL were

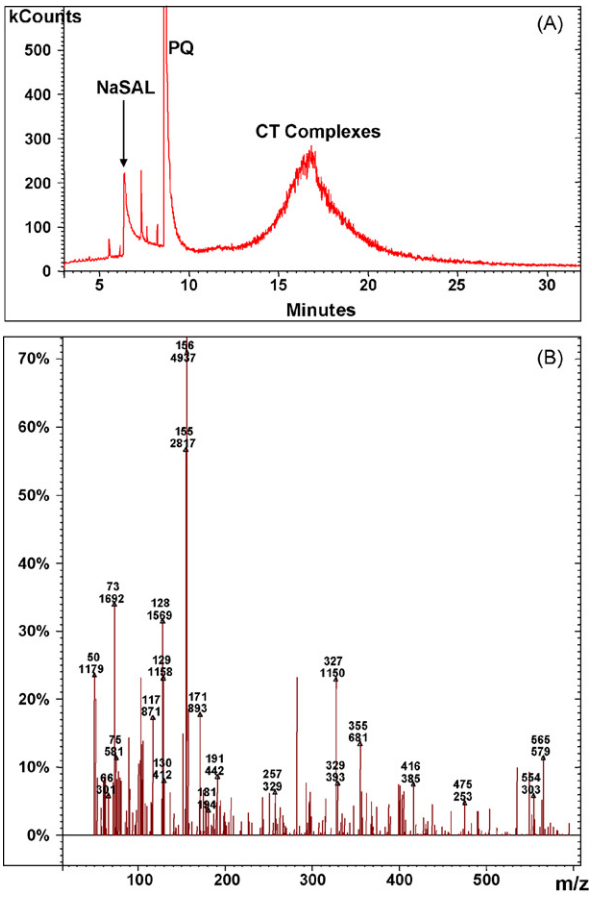


Fig. 7. (A) Gas chromatography–mass spectrometry–mass spectrometry spectrum of the complex obtained in FullScan mode. (B) Mass spectra obtained for the charge-transfer (CT) complexes methanol solution.

revealed by library matching using Saturn GC/MS/MS workstation software (NIST 05) and by comparison with the retention factors of PQ and NaSAL standards injected in the same chromatographic conditions. A completely different peak was observed when the methanol solution of the CT complexes was injected. The *m*/*z* of this unsharped peak (retention time of approximately 17 min) indicates an ion base with a *m*/*z* = 156 (characteristic of PQ fragmentation) and other higher *m*/*z* ion values (Fig. 7B). The identification of this chromatographic peak was not possible to obtain due to the limita-

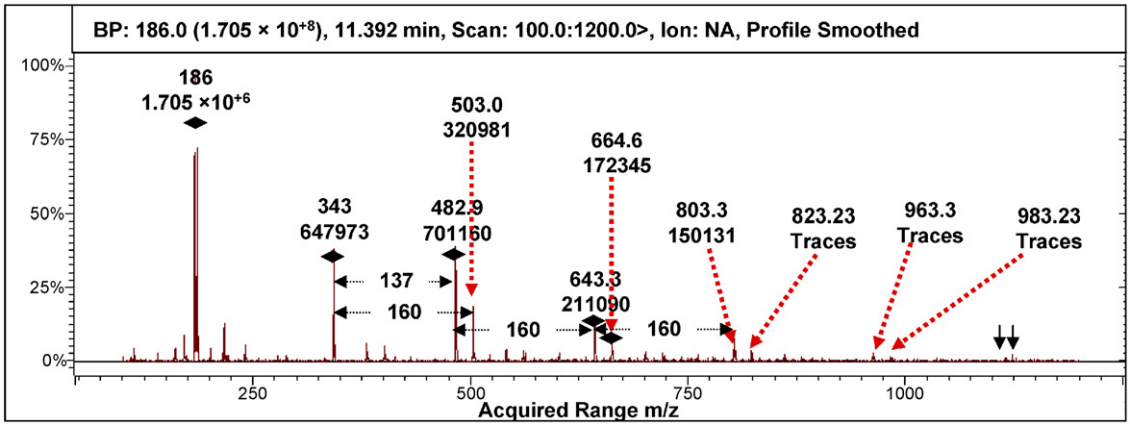


Fig. 8. Liquid chromatography/electrospray ionization/mass spectrometry/mass spectrometry spectrum of the charge-transfer (CT) complexes methanol solution acquired with only one voltage (10 V) in the Q1 chamber (correspond to one plan).

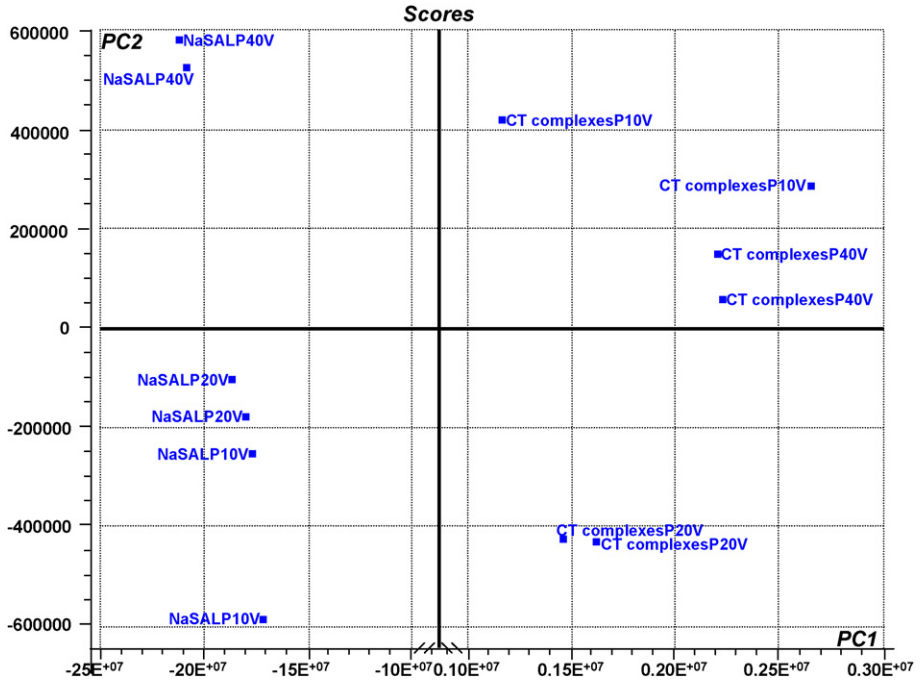


Fig. 9. Principal component analysis of each spectrum performed at different energies (1–100 V) in both methods: positive and negative CT complexes and sodium salicylate (NaSAL). Singular value decomposition—m/z: 240–1000, with leverage correction.

tion of using this technique for charged and high molecular weight compounds.

Liquid chromatography-mass spectrometry-mass spectrometry

ESI "fingerprint" mass spectra of pure standards of PQ and NaSAL, and of the CT complexes were collected under positive and negative ion mode. Pseudo-molecular ion, $[M-H]^-$ or $[M-H]^+$, was obtained as the base ion, respectively for NaSAL and PQ. The experimental result shows that NaSAL was easily protonated at the electrospray ion source under the experimental conditions, forming several positive ions, $[M-H]^+$. Therefore, electrospray in the positive ion mode was employed in this study. The CT complexes pseudo-molecular ions were investigated by direct infusion at low flow rate of 0.05 mL/min. Samples dissolved in methanol were injected into the ESI source, the capillary voltage was scanned as previously described and mass spectra were collected on single MS breakdown (Fig. 8). The CT complexes purity constituted another issue that needed to be dealt with. In fact the excess of NaSAL or PQ contribute with extra noise which hind the clear identification of the pseudo-molecular ion. Therefore multivariate analysis was carried out. The use of specific data mining algorithms is a standard procedure in spectroscopy analysis. The SVD was

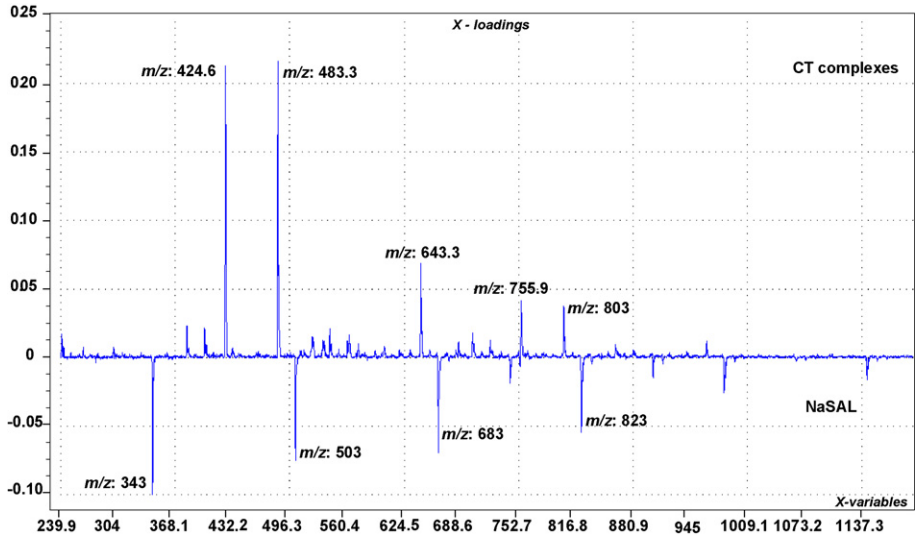


Fig. 10. Singular value decomposition—m/z: 240–1200, with leverage correction. Upper loadings: most relevant m/z of the CT complexes: 483 – PQ + 1 ($-OC_6H_4COO^-Na^+$) + 1 ($-OC_6H_4COOH$), 643 – PQ + 2 ($-OC_6H_4COO^-Na^+$) + 1 ($-OC_6H_4COOH$), 756 – PQ + 1 ($-OC_6H_4COO^-Na^+$) + 3 ($-OC_6H_4COOH$) and 803 – PQ + 3 ($-OC_6H_4COO^-Na^+$) + 1 ($-OC_6H_4COOH$). Lower loadings corresponds to most relevant m/z from NaSAL adducts (an ion formed by interaction of two species, usually an ion and a molecule, and often within the ion source, to form an ion containing all the constituent atoms of one species as well as an additional atom or atoms).

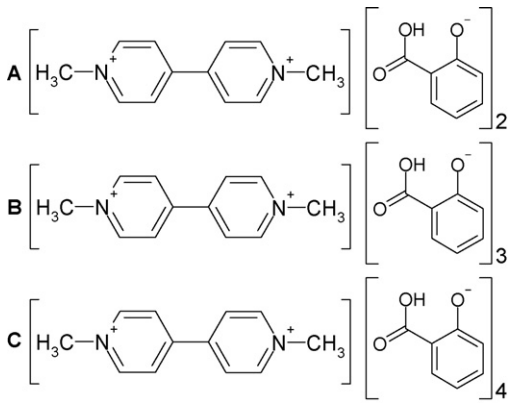


Fig. 11. Proposed structures of CT complexes formed between paraquat (PQ) and sodium salicylate (NaSAL).

employed to perform sample classification and, by studying the loadings, it is possible to select ions with the most probabilities to be the pseudo-molecular ion and after, validate the hypothesis by MS/MS fragmentation. The scores projections for the CT complexes and NaSAL samples obtained in positive mode are shown in Fig. 9, where a distinct separation between NaSAL and CT complexes was observed and the respective loadings are plotted in Fig. 10. After this "spectra filtration" the m/z identification was greatly simplified, which allowed to attain an ion profile of the CT complexes (Fig. 11). The elimination of noise obtained from the excess of NaSAL molecules, which constitute the impurity of the CT complexes molecules was attained. The detector was adjusted to measure m/z from 240 to 1200 in order to eliminate noise of the 160 (mass of NaSAL) and 137 (mass of salicylate ion). The scores projection of the samples in the new coordinate system provides "resolution" and the respective loadings calculated in SVD act as the "filtered" mass spectra (Fig. 10). They can be understood as the weights for each original variable (m/z) when calculating the principal component. The same principal m/z ions obtained were: 483, 643 and 803. In order to determine the pseudo-molecular fragmentation pattern of each possible fragment, selected by SVC analysis, the MS/MS technique was used. By setting the quadrupole (Q1) to pass only ions of interest (483, 643 and 803), the second quadrupole will act as a collision cell where the fragmentation occurs due to N2 mediator. The collision energy (between −5 and −40 V) was scanned using "Mass Breakdown" software feature. The third quadrupole acted as a secondary filter reducing chemical noise, leading to higher mass spectral sensitivity and selectivity. Results are shown in Table 1. A profile of fragmentation can be obtained for each ion. According to their fragmentations some structures are proposed (Fig. 11). In fact, all of them have a PQ molecule "linked" to one or more molecules of NaSAL. In all cases the carboxylic function is free or salified. It is the hydroxyl of the phenol function that interacts with the PQ molecule (Fig. 11). To keep each molecule without fragmentation, the collision energies must be lower than −5 V. Higher energies generate instability of the CT complexes and NaSAL molecules are dissociated of the CT complexes structures. Differences of m/z = 160 or 137 were observed when higher energies (more than −8.5 V) were applied.

Discussion and conclusions

According to previous studies (Dinis-Oliveira et al., 2008, 2007a,b) it has been clearly demonstrated that NaSAL confers a potent healing effect in the event of PQ-induced lung toxicity, which increases survival of intoxicated animals up to 100%. Several mechanisms involved in this healing effect were proposed and confirmed in pre-clinical approaches, namely the NaSAL-mediated modulation of pro-oxidant and pro-inflammatory pathways, as well as its anti-thrombogenic properties. In the present study a new, and most probably a relevant beneficial mechanism is presented, by demonstrating the chemical reactivity of PQ with NaSAL. The importance of the present results is further emphasised by the fact that NaSAL becomes the first therapeutic drug shown to react with PQ, which further endorses the possible treatment of PQ-poisoning patients with this antidote.

As demonstrated, by direct reaction with several compounds and by LC/ESI/MS/MS the hydroxyl group of NaSAL must be present to give the characteristic yellow colour solution. Furthermore, it is also noteworthy that besides phenol and NaSAL only lysine acetylsalicylate originated the same reactivity with PQ, in spite of having the hydroxyl function esterified. As it is well known, esterifications are highly reversible reactions as consequence of hydrolysis. Therefore salicylate anion is released and reacts with PQ. The lower hydrosolubility of the acetylsalicylic acid justifies the inability to

Table 2
Characterization of precursor's ions, product ions and structures of the CT complexes

Precursor	Product ion	CID, collision induced energy (V)	Structures	% of fragmentation	Ratio PQ/SAL	% of the different complexes found
803	803	−5 V	PQ + 3 ($-OC_6H_4COONa$) + 1 ($-OC_6H_4COOH$)	100	1/4	14
	186.1	−16 V	PQ	35.8		
	643.3	−8.5 V	PQ + 2 ($-OC_6H_4COONa$) + 1 ($-OC_6H_4COOH$)	4.4		
	664.6	−9.5 V	PQ + 3 ($-OC_6H_4COONa$)	1.74		
	483	−12 V	PQ + 1 ($-OC_6H_4COONa$) + 1 ($-OC_6H_4COOH$)	22.9		
	413	−16 V	No structure found			
643	643	−5 V	PQ + 2 ($-OC_6H_4COONa$) + 1 ($-OC_6H_4COOH$)	100	1/3	20
	483	−10 V	PQ + 1 ($-OC_6H_4COONa$) + 1 ($-OC_6H_4COOH$)	31.2		
	505.1	−9 V	PQ + 2 ($-OC_6H_4COONa$)	2.7		
	186.1	−14.5 V	PQ	31.83		
483	483	−5 V	PQ + 1 ($-OC_6H_4COONa$) + 1 ($-OC_6H_4COOH$)	100	1/2	66
	186.1	−10.5 V	PQ	57.4		
	186.2	−24 V	PQ	12.55		

CID, energies involved in the second quadrupole (Q2).

undergo hydrolysis and therefore to react with PQ. Observed CT complexes exhibited at least 3 colours: yellow, orange and red. A plausible explanation for the several colours could be given by the LC/ESI/MS/MS results, which revealed three major CT complexes (Figs. 8–11 and Table 2), corresponding to a stoichiometry of 1:2, 1:3 and 1:4 (PQ:NaSAL). Nevertheless it should not be excluded the possibility of occurring different complexations between PQ and NaSAL besides those selected by SVD sample classification, since other peaks of higher m/z were also recorded in LC/ESI/MS/MS analysis. Apparently, each cationic site in the bipyridylium salt could interact with several phenolic groups. As higher is the complexation rate more bathochromic shift could be observed and therefore CT complexes with yellow to red colours could be observed. This bathochromic shift was obvious when performing UV/vis spectrophotometry of the CT complex. The NaSAL 230 nm maximum was shifted to 239 nm (bathochromic effect) and the third maximum at 298 disappeared (Fig. 4A). Outside the linear range (Fig. 4B) absorbance between 350 and 550 nm (visible region) was only possible to observe in the CT complexes spectrum. Additional analytical findings corroborate the assumption that a new molecular entity ensues from mixing PQ and NaSAL, as revealed by a completely different m.p. of the CT complexes in comparison to isolated compounds. An advantage of using IR spectroscopy is related to the fact that if compounds did not react, the IR spectrum of the mixture should be the physical sum of the spectra of both compounds. As shown in Fig. 5 at least three changes were observed in the CT complex IR spectrum confirming that reactivity exists between PQ and NaSAL. In addition, by GC/MS/MS, a different peak appeared, although the identity was not possible to clarify (Fig. 7). Scanning electron photomicrographs strongly supported these results.

After these results, it is not overstressed to establish a strong relationship with some old publications on PQ field (Akhavein and Linscott, 1968; Ledwith and Woods, 1970; White, 1969). These investigators reported the CT interaction between PQ and various phenolic derivatives (electron donor molecules), which led to coloured solutions and clearly defined crystalline CT complexes. When a molecule of high electron affinity, such as NaSAL, aggregates with a molecule of relatively low ionization potential, such as PQ, there is a transfer of electronic charge from the donor molecule to the acceptor but no bonds are broken in either component. The resulting species is called CT complex. The appearance of a new electronic transition, upon mixing the components, without strong perturbation of the original spectroscopic transitions of each component as we observed by UV/vis, RMN and IR spectroscopy, is often taken as evidence for the existence of CT complexes. CT forces increase rapidly once the molecules involved have penetrated sufficiently close to overcome exchange repulsion forces within the van der Waals distances (<3 Å). Such penetration is allowed most easily in interactions involving planar lamellar configurations, as it is found in those involving PQ interactions. This ready formation of CT complexes was considered to be, possibly, one of the factors that contributes to resistance of plants against PQ toxicity (Ledwith and Woods, 1970; White, 1969). Their results show that PQ forms intermolecular complexes, exhibiting CT spectra, with a wide range of phenolic compounds (Gevao et al., 2000; Ledwith and Woods, 1970; Pacheco et al., 2003; White, 1969). Based on our results, NaSAL may possess a similar behaviour to that of polyphenols leading to the formation of CT complexes between the electron-poor pyridinium rings of PQ and the more electron-rich aromatic rings of NaSAL. The herbicidal efficacy of PQ is related to the concentration of free PQ^{2+} in solution inside the chloroplast and on the nature of the anion and any complexing agent with which it is applied (Homer and Tomlinson, 1959). Similarly to the plant kingdom, the same rational could be applied for the lungs in order to explain the full survival. Moreover, it may be postulated that the redox-cycle generated by PQ, which is responsible for its toxicity, may be affected since the complexation with one of the most powerful antioxidants, as is NaSAL, will definitely help in scavenging ROS (Dinis-Oliveira et al., 2006a,b,c,d,e; Graziano et al., 1974). Another interesting comparison could be made between our results and those of Silverman et al. (2005). These authors found that the addition of NaSAL to PQ spray solutions significantly decreased its herbicidal activity and concluded that a NaSAL-mediated pathway capable of protecting plants from reactive oxygen stress might be involved. Furthermore, it was also previously shown that endogenous salicylic acid protects rice plants from oxidative damage caused by PQ (Yang et al., 2004). The chemistry does certainly help to explain their findings.

An additional interesting finding of our study concerns to the low energy necessary to originate the CT complexes, since even in the solid state the complexation occurs, which is in accordance to the demands of CT complexes formation. Only one set of peaks was observed in the ^{1}H and ^{13}C NMR spectra of the complex of PQ with NaSAL, indicating fast-exchange complexation. Poulos et al. (1981) proposed that the rapid back-electron transfer prevents the effective build up of PQ free radical monocation (PQ^{+}) by returning the system to the ground state. If PQ^{+} is not generated no more ROS will be formed. Two properties, common to CT complexes formation and physiological processes, appear to be facile electron transfer and reversibility. Reversibility requires the free energy change involved in the interaction to be low and the activation energy to be small or zero. Facile electron transfer is attained when an acceptor donor interaction involves either donors with low ionization potentials, acceptors possessing high electron affinities, or both. Since CT complexes are formed so readily with PQ, it is reasonable to assume that they are involved in the transfer of an electron to PQ.

We had already proved that NaSAL treatment provided an effective inhibition of PQ-induced deleterious effects, namely oxidative stress, activation of transcription factors (NF-κB, p53, AP-1), platelet aggregation, cytochrome *c* release from mitochondria and consequent regulation of caspases activities. Importantly, this treatment was associated with a full survival of the PQ treated rats (extended for more than 30 days) in opposition to 100% of mortality by the day 6 in PQ-only exposed animals. Thus, NaSAL seems to be an effective antidote for PQ poisonings. Another important insight about the putative antidotal mechanisms and usefulness of salicylates, specially the water soluble forms, is now given. The next step is to perform detailed *in vivo* studies in order to access the kinetics of these CT complexes.

References

Akhavein, A.A., Linscott, D.L., 1968. The dipyridylium herbicides, paraquat and diquat. Residue Rev. 23, 97–145.

Brunton, L., Lazo, J., Parker, K., 2006. Goodman & Gilman's The Pharmacological Basis of Therapeutics. McGraw Hill, New York.

Dinis-Oliveira, R.J., Duarte, J.A., Remião, F., Sanchez-Navarro, A., Bastos, M.L., Carvalho, F., 2006a. Single high dose dexamethasone treatment decreases the pathological effects and increases the survival rat of paraquat-intoxicated rats. Toxicology 227, 73–85.

Dinis-Oliveira, R.J., Remião, F., Duarte, J.A., Sanchez-Navarro, A., Bastos, M.L., Carvalho, F., 2006b. P-glycoprotein induction: an antidotal pathway for paraquat-induced lung toxicity. Free Radic. Biol. Med. 41, 1213–1224.

Dinis-Oliveira, R.J., Remião, F., Duarte, J.A., Sanchez-Navarro, A., Bastos, M.L., Carvalho, F., 2006c. Paraquat exposure as an etiological factor of Parkinson's disease. Neurotoxicology 27, 1110–1122.

Dinis-Oliveira, R.J., Sarmento, A., Reis, P., Amaro, A., Remião, F., Bastos, M.L., Carvalho, F., 2006d. Acute paraquat poisoning: report of a survival case following intake of a potential lethal dose. Pediatr. Emerg. Care 22, 537–540.

Dinis-Oliveira, R.J., Valle, M.J., Bastos, M.L., Carvalho, F., Sanchez-Navarro, A., 2006e. Kinetics of paraquat in the isolated rat lung: influence of sodium depletion. Xenobiotica 36, 724–737.

Dinis-Oliveira, R.J., Sousa, C., Remião, F., Duarte, J.A., Sanchez-Navarro, A., Bastos, M.L., Carvalho, F., 2007a. Full survival of paraquat-exposed rats after treatment with sodium salicylate. Free Radic. Biol. Med. 42, 1017–1028.

Dinis-Oliveira, R.J., Sousa, C., Remião, F., Duarte, J.A., Ferreira, R., Sánchez-Navarro, A., Bastos, M.L., Carvalho, F., 2007b. Sodium salicylate prevents paraquat-induced apoptosis in the rat lung. Free Radic. Biol. Med. 43, 48–61.

Dinis-Oliveira, R.J., Remião, F., Duarte, J.A., Sanchez-Navarro, A., Bastos, M.L., Carvalho, F., 2008. Paraquat poisonings: mechanisms of lung toxicity, clinical features, and treatment. Crit. Rev. Toxicol. 38, 13–71.

Gevao, B., Semple, K.T., Jones, K.C., 2000. Bound pesticide residues in soils: a review. Environ. Pollut. 108, 3–14.

Graziano, J.H., Grady, R.W., Cerami, A., 1974. The identification of 2,3-dihydroxybenzoic acid as a potentially useful iron-chelating drug. J. Pharmacol. Exp. Ther. 190, 570–575.

Homer, R.F., Tomlinson, T.E., 1959. Redox properties of some dipyridyl quaternary salts. Nature 184, 2012–2013.

Ledwith, A., Woods, H.J., 1970. Charge transfer spectra and reaction intermediates. Part II: Stable crystalline complexes from phenols and *N*,*N*-dimethyl-4,4-bipyridylium (paraquat) salts. J. Chem. Soc. C, 1422–1425.

Pacheco, M.L., Peña-Méndez, E.M., Havel, J., 2003. Supramolecular interactions of humic acids with organic and inorganic xenobiotics studied by capillary electrophoresis. Chemosphere 51, 95–108.

Poulos, A.T., Kelly, C.K., Simone, R., 1981. Photoinduced electron transfer in the *p*-phenylenediamine-paraquat molecular complex. J. Phys. Chem. 85, 823–826.

Silverman, F.P., Petracek, P.D., Fledderman, C.M., Ju, Z., Heiman, D.F., Warrior, P., 2005. Salicylate activity. 1: Protection of plants from paraquat injury. J. Agric. Food Chem. 53, 9764–9768.

White, B.G., 1969. Bipyridylium quaternary salts and related compounds. III: Weak intermolecular charge-transfer complexes of biological interest, occurring in solution and involving paraquat. Trans. Faraday Soc. 65, 2000–2015.

Yang, Y., Qi, M., Mei, C., 2004. Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. Plant J. 40, 909–919.