Chemotherapy-Induced Pulmonary Hypertension

Role of Alkylating Agents

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Pulmonary veno-occlusive disease (PVOD) is an uncommon form of pulmonary hypertension (PH) characterized by progressive obstruction of small pulmonary veins and a dismal prognosis. Limited case series have reported a possible association between different chemotherapeutic agents and PVOD. We evaluated the relationship between chemotherapeutic agents and PVOD. Cases of chemotherapy-induced PVOD from the French PH network and literature were reviewed. Consequences of chemotherapy exposure on the pulmonary vasculature and hemodynamics were investigated in three different animal models (mouse, rat, and rabbit). Thirty-seven cases of chemotherapy-associated PVOD were identified in the French PH network and systematic literature analysis. Exposure to alkylating agents was observed in 83.8% of cases, mostly represented by cyclophosphamide (43.2%). In three different animal models, cyclophosphamide was able to induce PH on the basis of hemodynamic, morphological, and biological parameters. In these models, histopathological assessment confirmed significant pulmonary venous involvement highly suggestive of PVOD. Together, clinical data and animal models demonstrated a plausible cause-effect relationship between alkylating agents and PVOD. Clinicians should be aware of this uncommon, but severe, pulmonary vascular complication of alkylating agents. (Am J Pathol 2015, 185: 356–371; http://dx.doi.org/10.1016/j.ajpath.2014.10.021)
such as bleomycin, carmustine, and mitomycin, have been reported to be associated with PVOD. Moreover, bone marrow transplantation (BMT) is also considered to be a risk factor for the development of PVOD. Despite the recognition that chemotherapy can potentially provoke PVOD, no specific drug or therapeutic class has been clearly identified as a risk factor for PVOD.

The first objective of the present study was to determine the chemotherapeutic agents that could be involved in the development of PVOD. Thus, we reviewed cases of chemotherapy-induced PVOD from the French PH network and performed a systematic literature review to identify potential chemotherapeutic agents implicated in the development of PVOD. Furthermore, additional treatment, including radiotherapy and the role of BMT, was investigated. Herein, we demonstrate that alkylating agents are the predominant chemotherapeutic class associated with PVOD, with cyclophosphamide (CP) being detected in nearly half of all chemotherapy-induced PVOD cases. On the basis of this finding, we investigated the consequences of CP exposure on the pulmonary vasculature and hemodynamics in three different animal models (mouse, rat, and rabbit). The influences of sex, time course, and dose-response to CP were also evaluated. We analyzed right heart and pulmonary artery (PA) hemodynamics, morphological parameters (in heart and lungs), and biological parameters (in lungs and serum/blood cells) relevant to pulmonary vascular dysfunction and inflammation. Finally, we performed in vivo experiments on whether cytotoxic agents (mesna and amifostine) used during treatment with CP could prevent the development of PH.

Materials and Methods

Identified Cases of Chemotherapy-Related PVOD in the French PH Network and in the Literature

We reviewed all cases from the French PH Registry of confirmed precapillary PH among patients in whom PVOD was suspected after commencement of chemotherapy, radiotherapy, and/or BMT.

The French PH Registry was established in accordance with French bioethics laws (National Commission on Informatics and Liberty) in 2004, and all included patients gave their informed consent.

Routine evaluation at baseline included demographics, medical history, physical examination, echocardiography, chest high-resolution computed tomography, ventilation/perfusion lung scan, abdominal ultrasound, autoimmunity screening, and HIV serological test. Precapillary PH was confirmed in all cases by right heart catheterization (RHC). At diagnosis, New York Heart Association functional class and 6-minute walk distance were recorded.

Data were centrally collected and analyzed at the French referral center for severe PH (Hôpital Bicêtre, University Paris-Sud, Le Kremlin-Bicêtre, Paris, France).

The systematic literature review in PubMed and Medline was performed on December 1, 2013. Key words entered in the medical subject headings (MESH) database were as follows: cancer [MESH] and pulmonary veno-occlusive disease, radiotherapy [MESH] and pulmonary veno-occlusive disease and chemotherapy [MESH], and pulmonary veno-occlusive disease. The chosen key words cancer, chemotherapy, and radiotherapy [MESH] were also crossed to pulmonary capillary hemangiomatosis, an entity considered to be similar to PVOD. In addition, we crossed specific chemotherapeutic agents implicated in the development of PVOD, including cisplatin, cyclophosphamide, and mitomycin [MESH] with pulmonary veno-occlusive disease. With respect to different search terms, the following number of articles were retrieved: cancer [MESH] and pulmonary veno-occlusive disease (n = 115), radiotherapy [MESH] and pulmonary veno-occlusive disease (n = 14), chemotherapy [MESH] and pulmonary veno-occlusive disease (n = 48), cancer [MESH] and pulmonary capillary hemangiomatosis (n = 62), chemotherapy [MESH] and pulmonary capillary hemangiomatosis (n = 2), and radiotherapy [MESH] and pulmonary capillary hemangiomatosis (n = 0). Search terms containing specific chemotherapeutic agents yielded the following: cisplatin [MESH] and pulmonary veno-occlusive disease (n = 3), cyclophosphamide [MESH] and pulmonary veno-occlusive disease (n = 21), and mitomycin [MESH] and pulmonary veno-occlusive disease (n = 3).

Analysis of Case Reports

All identified case reports were adjudicated to determine whether a diagnosis of PVOD was compatible. This was on the basis of functional, radiological, hemodynamic, and histological data (if available) presented in each case report. Functional evaluation was on the basis of reported worsening dyspnea, as determined by the New York Heart Association functional class; radiological assessment included chest radiography and/or chest high-resolution computed tomography; and hemodynamic evaluation included echocardiographic examination and/or RHC. Two pneumologists (S.G. and D.M.) reviewed all case reports independently, and one pathologist specializing in pulmonary vascular diseases (P.D.) reviewed all histological data. Final decision on the classification of PVOD was reached by consensus, and patients were classified into the following three groups: i) confirmed PVOD, ii) highly probable PVOD, and iii) probable PVOD. Criteria for confirmed PVOD required histological proof of PVOD by either lung biopsy or post-mortem examination. Patients were considered to have highly probable PVOD if RHC confirmed precapillary PH in association with consistent radiological findings. Diagnosis of probable PVOD was given even in the absence of a histological proof or the presence of PH on RHC, on the basis of clinical presentation and functional evaluation, including echocardiography.

Detailed information was recorded concerning administered chemotherapeutic agents, including frequency and duration of exposure. Each chemotherapeutic agent was recorded once, even if administrated several times during the
course of treatment, and the agents were classified according to their therapeutic group. Other treatments consisted of radiotherapy and BMT, including graft-versus-host disease prophylaxis. The site, cumulative dose (in Gy), and frequency of radiotherapy were noted. Similarly, drugs used for graft-versus-host disease prophylaxis were recorded once even if administrated several times in the course of treatment.

Chemotherapies and Associated Therapy (Radiotherapy and BMT)

All patients were treated with various chemotherapeutic regimens, depending on the type, stage, and severity of underlying cancer. In addition, frequency of chemotherapy, dose administration, and each cycle of chemotherapy were different. Therefore, we decided to note each substance only once, even if administrated several times in the course of treatment.

In Vivo Study Design

We exploited three different animal models (mice, rats, and rabbits) to assess the pathobiological characteristics of the in vivo models of CP-induced PVOD. Rodents have muscular pulmonary veins, whereas human pulmonary veins contain thin fibrous walls. Given the greater similarity in the structure of pulmonary veins between rabbits and humans, we also injected rabbits with CP (Endoxan; Baxter, Deerfield, IL) to compare the histopathological features of PVOD between human and animal after alkylating agent exposure.

Mice and rats were housed at the Faculty of Pharmacy of Châtenay-Malabry (ANIMEX platform, Châtenay Malabry, France). Experiments were conducted according to the European Union regulations (European Economic Community Directive 86/609) for animal experiments and complied with our institution’s guidelines for animal care and handling. The animal facility is licensed by the French Ministry of Agriculture (agreement number B92-019-01). This study was approved by the Committee on the Ethics of Animal Experiments CEEA26 CAP Sud. Mouse and rat experiments were supervised by F.P. (agreement delivered by the French Ministry of Agriculture for animal experiment number A92-392). All efforts were made to minimize animal suffering. Rabbits were housed at the Catholic University of Leuven (KU Leuven, Leuven, Belgium). The study of the potential toxic effects of CP on the rabbit pulmonary vasculature was approved by the Animal Ethics Committee of the KU Leuven (license number LA1210263). All animals were maintained in a temperature-controlled room with a 12/12-hour light/dark cycle.

<table>
<thead>
<tr>
<th>Characteristics</th>
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<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
</tr>
</thead>
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<td>50</td>
<td>53</td>
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<tr>
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<td>Female</td>
<td>Female</td>
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<td>Mitomycin</td>
</tr>
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<td>IV</td>
<td>III</td>
<td>IV</td>
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<tr>
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<td>238</td>
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<td>74</td>
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<td>Yes</td>
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<td>Ground-glass opacities (yes or no)</td>
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<td>No</td>
<td>Yes</td>
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</tbody>
</table>

CI, cardiac index; CO, cardiac output; DLCO, diffusing capacity of the lung for carbon monoxide; FC, functional class; FEV₁, forced expiratory volume in 1 second; HRCT, high-resolution computed tomography; mPAP, mean pulmonary arterial pressure; 6-MWD, 6-minute walk distance; NA, not available; PaO₂, partial pressure of arterial oxygen; PCWP, pulmonary capillary wedge pressure; PH, pulmonary hypertension; PVOD, pulmonary veno-occlusive disease; PVR, pulmonary vascular resistance; WHO, World Health Organization.
Mice
Male and female C57BL/6J mice (Janvier, 7 to 10 weeks old) were randomly divided into saline-treated control group (control; \( n = 17 \)) and CP-exposed group (350 mg/kg i.p., CP; \( n = 60 \)). We performed hemodynamic measurements and tissue collection 4 weeks after injection.

Rats
Male and female Wistar rats (Janvier, 8 weeks old) were subject to different protocols to evaluate sex differences, kinetics, dose-response relationship, and effects of cytoprotective agents on CP exposure. Both male and female rats were used in protocol 1 (see below). Subsequently, only females were used because of their higher susceptibility to the development of PH after CP injection (protocols 2 to 5).

Protocol 1. Sex-specific susceptibility. Male and female rats were randomly divided into saline (control, \( n = 5 \)) or CP-exposed groups [Endoxan (Baxter), 350 mg/kg, i.p.,

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Table 1 (continued)

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Daunorubicin, cytarabine, gemtuzumab ozogamicin, fludarabine, mycophenolate mofetil, and cyclosporine

<table>
<thead>
<tr>
<th></th>
<th>Mitomycin and fluorouracil</th>
<th>Mitomycin and fluorouracil</th>
<th>Cisplatin and docetaxel</th>
<th>Mitomycin</th>
<th>Docetaxel and doxorubicin</th>
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<td>III</td>
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Figure 1  Histological characteristics from a patient with cyclophosphamide-associated pulmonary veno-occlusive disease (PVOD). A: Septal vein displaying significant muscularization and intimal thickening. B: Small preseptal vein with near-occlusive collagen-rich fibrosis. C: Numerous microvessels (arterioles or venules) show significant concentric muscularization. Note accumulations of siderin-laden intra-alveolar macrophages. D: Pulmonary artery (with adjacent bronchiole) displaying significant remodeling, more precisely hypertrophy of the media and intimal fibrosis. A–D: Hematoxylin and eosin stain. E and F: High-resolution computed tomography of the chest showing septal lines and centrilobular ground-glass opacities, suggestive of PVOD.
We performed hemodynamic measurements, right ventricle hypertrophy (RVH) assessment, and tissue collection 4 weeks after injection.

**Protocol 2.** Kinetics of PH development. Female rats were randomly divided into saline (control, \( n = 10 \)) or CP-exposed groups (350 mg/kg, i.p., \( n = 40 \)). We performed hemodynamic measurements, RVH assessment, and tissue collection at 1, 2, 3, and 4 weeks after CP injection (\( n \geq 6 \) to 10 rats for each time point).

**Protocol 3.** Dose-response relationships. Female rats were randomly divided into saline (control, \( n = 5 \)) or CP-exposed groups: 100, 150, 200, and 250 mg/kg per week/2 weeks (\( 2 \times 100, 2 \times 150, 2 \times 200, \) and \( 2 \times 250 \), respectively; \( n = 5 \) for each dose) with sacrifice at 4 weeks after the second injection for hemodynamic measurements, RVH assessment, and tissue collection. No rat survived to 4 weeks at the peak dose of 250 mg/kg per week/2 weeks (\( 2 \times 250 \)).

**Protocol 4.** Prevention study. Amifostine was given i.p. at 200 mg/kg 30 minutes before 200 mg/kg per week/2 weeks of CP (\( n = 20 \)). Mesna was given as i.p. injections at a dosage equal to 20% of CP dose at the time of administration, 4 hours after each CP dose, and 8 hours after each CP dose (\( n = 20 \)). These two groups were compared to saline (control, \( n = 10 \)) and CP only—exposed (CP, 200 mg/kg per week/2 weeks, \( n = 20 \)) groups. Survival was evaluated over 4 weeks in the three CP-exposed groups.

### Table 2  Identified Cases of Chemotherapy-Induced Pulmonary Veno-Occlusive Disease in the Literature with Detailed Information about Age, Sex, Underlying Malignancies, and Type of Chemotherapy

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Type of cancer</th>
<th>Chemotherapy</th>
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</thead>
<tbody>
<tr>
<td>12</td>
<td>42</td>
<td>F</td>
<td>Hodgkin lymphoma</td>
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<td>10</td>
<td>12</td>
<td>M</td>
<td>Acute lymphoblastic leukemia</td>
<td>Vincristine, cyclophosphamide, daunorubicin, and cytarabine</td>
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<tr>
<td>13</td>
<td>24</td>
<td>M</td>
<td>Acute lymphoblastic leukemia</td>
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<tr>
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<td>M</td>
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<td>36</td>
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<td>Lung adenocarcinoma</td>
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<td>63</td>
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<td>Lung adenocarcinoma</td>
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<td>Lymphocytic lymphoma</td>
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</tr>
</tbody>
</table>

F, female; M, male.
Survivors were sacrificed 4 weeks after the second CP injection for hemodynamics, RVH measurements, and tissue collection.

Protocol 5. Long-term CP exposure. We used a lower dose of CP (100 mg/kg), allowing repeated injections of CP without premature death because of early heart failure. CPs were injected i.p. at 0, 1, 5, 6, 10, and 11 weeks and sacrificed at 13 weeks after the start of this study (n = 6).

Rabbits
Adult female New Zealand rabbits (2.5 to 3 kg) were obtained from the animal facility (KU Leuven). All rabbits were housed individually (surface, 0.36 m²) under controlled conditions: room temperature of 18°C to 21°C, 12/12-hour light/dark cycle, free access to water, standard rabbit chow once a day, and dry grass once a week.

CP injection: Preoperative analgesia was induced by an i.m. injection of 25 mg/kg ketamine (Anesketin; Eurovet, Heusden-Zolder, Belgium) and 0.5 mg/kg medetomidine (Domitor; Janssen Pharmaceutica, Beerse, Belgium). During the entire procedure, animals were supplied with oxygen through a mask, maintained on a heating pad, and heart rate and blood oxygen saturation were continuously monitored using a pulse oximeter (GE Carescape V100; Uno, Zavenaar, the Netherlands) placed over the right ear central artery. Arterial blood was collected from the ear central artery, and plasma was isolated and stored at −80°C. CP (100 mg/kg) reconstituted in 0.9% NaCl, previously warmed at 37°C, was injected i.p. Medetomidine-induced sedation was reversed by an i.m. injection of 1 mg/kg atipamezole (Antisedan; Elanco, Brussels, Belgium). When eye and pedal reflexes were observed, the animals were returned to their individual cages.

Rabbits were injected with CP (n = 6) or saline (n = 4) at 0, 1, and 3 weeks and sacrificed at 8 weeks after the first injection for RVH measurement and tissue collection.
Hemodynamic Measurements, RVH Evaluation, and Tissue Collection

Mice and rats were both anesthetized with 2 L/minute O₂/3% isoflurane (Minerve, Esternay, France). Hemodynamic measurements were recorded with a PowerLab 4/35 data acquisition system and analyzed with LabChart 7 software version 7.3.1 (ADInstruments, Oxford, UK).

Right ventricle systolic pressure was measured in anesthetized mice by transthoracic puncture.

In anesthetized rats, ventral tail artery blood sampling for serum collection was performed before hemodynamic measurements. A 3.5-French umbilical vessel catheter (Tyco, Plaisir, France) was introduced into the right external jugular vein. With the angle directed anteriorly, the catheter was inserted 2.5 cm proximally into the right atrium. The catheter was then rotated 90 degrees anticlockwise and advanced 1 cm distally into the right ventricle (RV) and finally into the PA after an additional 1.5-cm advancement. Correct anatomical placement was confirmed by respective pressure contours. A T-type Ultra Fast Thermocouple Probe (IT-23; ADInstruments, Oxford, UK) was inserted into the left carotid artery to allow measurement of cardiac output (CO) by the thermodilution technique, after injection of cold saline into the PA. Data of mean PA pressure (in mmHg), CO (in mL/minute), and total pulmonary resistances (TPRs; in mmHg/mL per minute) were subject to statistical analyses.

After exsanguination of animals (mice, rats, and rabbits), the hilum of the left lung was ligated and the right lung was distended by infusion of formalin via the trachea, and then embedded in paraffin. The noninflated left lung was snap frozen in liquid nitrogen and used for protein and mRNA extractions and quantifications. For Fulton’s index of RVH, the ratio of right ventricle (RV)/body weight significantly increases (C), whereas the ratio of LV/body weight is unchanged (D). B–D: Control, n = 17; CP-exposed mice, n = 49. Four weeks after CP exposure, PH is associated with foci of septal thickening and accumulation of large foamy intra-alveolar macrophages (E) that are absent in nonexposed mice (F) (hematoxylin and eosin). *P < 0.05, ****P < 0.0001.

Quantification of Pulmonary Microvessel Neomuscularization

Paraffin-embedded lungs were divided into sections (5 μm thick) and stained in double immunofluorescence, for von Willebrand factor (vWF; Dako, Glostrup, Denmark) and α-smooth muscle actin (α-SMA; clone 1A4; Sigma-Aldrich, Lyon, France). Pulmonary microvessels (40 to 60; <50 μm thick) were analyzed and categorized as muscularized when vWF⁺ endothelial cells in microvessels were coated with α-SMA⁺ smooth muscle cells when vessels contained at least one cell that was positive for α-SMA but lacked a continuous layer or nonmuscularized (NM) in the absence of this coating, to assess the degree of muscularization of these normally NM precapillary and post-capillary vessels.
Hematein-eosin-safran and orcein stains were applied on paraffin lung sections (5 μm thick) following routine procedure.

Flow Cytometry

A no-lyse no-wash immunophenotyping of blood leukocytes was performed during the kinetics of PH development after single exposure to CP in rats with the triple-color reagent (CD3/CD45RA/CD161) from AbD Serotec (Oxford, UK). Flow cytometry was performed on a MACSQuant analyzer (Miltenyi, Paris, France). During the acquisition, nucleated blood leukocytes were gated in the Hoechst-positive fraction of events. The data were analyzed using the MACSQuantify software version 2.5 (Miltenyi).

Multiplex Data

We performed simultaneous quantification of recognized serum biomarkers of cardiovascular diseases in sera from control and CP-exposed rats with Millipore’s MILLIPLEX (Millipore, Molsheim, France). The MILLIPLEX MAP Rat Cardiovascular Panel 1 (Millipore) was used for the simultaneous quantification of B-type natriuretic peptide, IL-6, monocyte chemoattractant protein-1, myeloperoxidase, plasminogen activator inhibitor 1, tissue inhibitor of matrix metalloproteinases type I, tumor necrosis factor α, troponin I, troponin T, vascular endothelial growth factor, and vWF. The MILLIPLEX MAP Rat Cardiovascular Panel 2 (Millipore) was used for the simultaneous quantification of soluble E-selectin and soluble intercellular adhesion molecule 1. MILLIPLEX plates were washed with a Bio-Plex Pro II Wash Station (Biorad, Marnes-la-Coquette, France) and read with a Luminex 200 (Millipore). Data were analyzed with the Bioplex manager software version 6.1 (Biorad). MILLIPLEX experiments were performed at INSERM IFR 65 (Saint Antoine Hospital, Paris, France).

Western Blot Analysis

Lung tissue samples from rat were prepared in lysis buffer containing 1% Igepal, 20 mmol/L Tris-HCl, 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L Na3VO4, 10 μg/μL leupeptin, 10 μg/μL pepstatin A, 10 μg/μL aprotinin, and PefaBloc protease inhibitor cocktail (aprotinin, leupeptin, and PefaBloc; Roche, Meylan, France; other lysis buffer components come from Sigma-Aldrich). Protein lysates [40 μg for the potassium channel subfamily K, member 3 (KCNK3), and 10 μg for CD45 detection] were separated onto SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking, membranes were incubated in Tris-buffered saline and Tween 20 and 5% nonfat milk overnight at 4°C with primary antibodies: rabbit anti-KCNK3 (dilution 1:2000), mouse anti-CD45 (dilution 1:300), mouse anti-CD2 (dilution 1:10,000; Cell Signaling, Danvers, MA) or with horseradish peroxidase—conjugated goat anti-rabbit (dilution 1:10,000; Cell Signaling, Danvers, MA) or with horseradish peroxidase—conjugated goat anti-mouse (dilution 1:10,000; Cell Signaling, Danvers, MA) or with horseradish peroxidase—conjugated goat anti-rabbit (dilution 1:5000; Cell Signaling, Danvers, MA) or with horseradish peroxidase—conjugated goat anti-rabbit (dilution 1:5000; Cell Signaling), accordingly. Blots were revealed using electrochemiluminescence reagents (Perkin Elmer, Villebon sur Yvette, France). ImageJ software version 1.48 (NIH, Bethesda, MD) was used to quantify the level of protein expression.

Determination of Pulmonary Serotonin Levels Using Liquid Chromatography/Tandem Mass Spectrometry

Preparation of Standards

Stock solutions of serotonin (Sigma-Aldrich) and serotonin-D4, used as internal standards (Cluzeau Info Labo, Sainte-Foy-La-Grande, France), were prepared at a concentration of 10 mg/mL in distilled water. They were then diluted as working solutions in 100 mmol/L ammonium formate (buffered with formic acid at pH = 3.5) at concentrations of 1 and 3 μg/mL, respectively. The internal standard was included in the sample before extraction.
quadruple mass spectrometer (Waters, Milford, MA) using both serotonin and serotonin-D4. The retention time was 1 minute for each sample. The retention time was 1 minute for pulmonary resistances (TPRs; in mmHg/mL per minute). * Pressure (mPAP; in mmHg).

Figure 4  Cyclophosphamide (CP) induces pulmonary hypertension (PH) in a dose-dependent manner. Dose-response study with fractioned lower doses of CP in female rats: 100, 150, 200, and 250 mg/kg per week for 2 weeks (2 × 100, 2 × 150, and 2 × 200, respectively), with sacrifice 4 weeks after the second injection. No rat survived to 250 mg/kg per week for 2 weeks (2 × 250) until 4 weeks. Hemodynamic parameters and right heart hypotrophy were evaluated. A: Fulton index (right ventricle (RV)/left ventricle (LV) + septum (S) weight ratio]. B: Mean pulmonary artery pressure (mPAP; in mmHg). C: Cardiac output (CO; in mL/minute). D: Total pulmonary resistances (TPRs; in mmHg/mL per minute). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Sample Preparation for Liquid Chromatography/Tandem Mass Spectrometry

The extraction solution was composed of 10% 100 mmol/L ammonium formate (pH 3.5), 10% 3 μg/mL serotonin-D4 solution, and 80% acetonitrile, allowing metabolite extraction and protein precipitation. Pieces of frozen lungs from rats were weighed (approximately 50 mg) and homogenized into 1 mL of extraction solution using GentleMACS dissociator (Miltenyi Biotech, Paris, France). The homogenates were then centrifuged at 1500 × g for 5 minutes at 4°C to discard macroscopic residues. The supernatants were collected and submitted to two successive centrifugations at 15,000 × g for 10 minutes at 4°C to further eliminate small residues. Supernatants were collected after each centrifugation to achieve a clear solution and then stored at −20°C until use.

Liquid Chromatography/Tandem Mass Spectrometry Data

Each standard (serotonin and serotonin-D4 solutions) and extracts (10 μL) were injected in a high-performance liquid chromatographic instrument (Ultimate 3000 DGP3600; Dionex, Sunnyvale, CA) with an automatic injector WPS 3000 set at 4°C. Separation of metabolites was performed using a Nucleoshell Hydrophilic Interaction Liquid Chromatography (HILIC) column (100 × 2 mm, 2.7 μm particle size; Macherey-Nagel, Hoerd, France). The mobile phase, composed of acetonitrile/100 mmol/L ammonium formate (pH 3.5), 80/20 (v/v), was used at a flow rate of 400 μL/minute. The run time was 4.5 minutes for each sample. The retention time was 1 minute for both serotonin and serotonin-D4.

Detection of serotonin was achieved with a triple-quadruple mass spectrometer (Waters, Milford, MA) using an electrospray ionization source. The source and desolvation gas temperatures were set to 120°C and 350°C, respectively. Desolvation gas flow was set to 479 L/hour; and capillary and cone voltages, 2.5 kV and 35 V, respectively. Multiple reaction monitoring was used in positive mode to detect precursor ions of serotonin at m/z 177 and serotonin-D4 at m/z 181. Applying collision energies of 10 and 11 eV, product ions were obtained at m/z 160 and 164 for serotonin and serotonin-D4, respectively.

Semiquantitative Analysis

Peaks of serotonin and internal standard serotonin-D4 were integrated, and the variability of extraction rates was corrected, calculating the ratio between peak areas of serotonin and serotonin-D4. Then, these ratios were divided by the weight of lung pieces.

Plasma Endothelin Quantification by Enzyme-Linked Immunosorbent Assay

Endothelin-1 levels were measured in rabbit plasma samples using a QuantiGlo Endothelin-1 immunoassay, per the manufacturer’s instructions (R&D Systems, Lille, France).

Statistical Analysis

Unless otherwise expressed, quantitative variables were presented as means ± SEM. Between groups, comparisons were made with paired or unpaired Student’s t-test or Mann-Whitney test (comparison between two groups), analysis of variance, or Kruskal-Wallis test (comparison between more than two groups), followed by Tukey’s or Dunn’s test,

Figure 5  Dose-response study with fractioned lower doses of cyclophosphamide in female rats: 100, 150, 200, and 250 mg/kg per week for 2 weeks (2 × 100, 2 × 150, and 2 × 200, respectively), with sacrifice 4 weeks after the second injection. No rat survived to 250 mg/kg per week for 2 weeks (2 × 250) until 4 weeks. Muscularization of distal vessels (DVs; <50 μm) was evaluated. Percentage of nonmuscularized (NM) DVs (A), partially muscularized (PM) DVs (B), fully muscularized (FM) DVs (C), and occluded DVs (D). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
respectively, according to the normality of the distribution. Survival was compared using the Mantel-Haenszel log-rank test. \( P < 0.05 \) was considered statistically significant.

Results

Characteristics of Suspected Chemotherapy-Related PVOD in the French PH Network and Systematic Review of the Literature

In the French PH network, we identified 10 cases of chemotherapy-induced PVOD in the setting of anal cancer \((n = 4)\), breast cancer \((n = 2)\), lung cancer \((n = 1)\), Hodgkin lymphoma \((n = 2)\), and acute myeloblastic leukemia \((n = 1)\). Demographic, functional, and hemodynamic characteristics of these patients are detailed in Table 1. Histological confirmation of PVOD after treatment with chemotherapy was obtained in one patient (Figure 1). Regarding the systematic literature review, we identified 179 eligible articles. After the adjudication process (online methods), a final consensus decision on the classification of PVOD was reached on the basis of clinical, functional, hemodynamic, radiological, and histological findings. Twenty-seven patients from 179 articles were considered to represent chemotherapy-induced PVOD. Histological data were available in 22 (81.5%) of 27 patients either by lung biopsy or autopsy, of which 20 (74.1%) of 27 cases were classified as confirmed PVOD on the basis of compatible histological descriptions. The diagnosis of highly probable PVOD, on the basis of RHC and characteristic radiological signs on chest high-resolution computed tomography, was assigned to 4 (14.8%) of 27 patients and probable PVOD was attributed to the remaining 3 (11.1%) of 27 patients. Table 2 shows the demographic characteristics, type of underlying cancer, and chemotherapeutic regimen of the 27 PVOD patients identified in the literature. Combining all identified cases from the French PH network and literature review \((n = 37)\), the median age of patients with chemotherapy-induced PVOD was 37.8 years (range, 2 to 66 years). No sex predominance was observed (45.9% males versus 54.1% females).

Regarding the underlying type of cancer, we separated solid malignancies from hematological disorders. Identified solid malignancies observed in the setting of developed PVOD were breast cancer \((n = 2)\), brain malignancies \((n = 3)\), including neuroblastoma \((n = 1)\), astrocytoma \((n = 1)\), and glioma \((n = 1)\), cervical carcinoma \((n = 2)\), lung cancer \((n = 4)\), represented by different histological types [adenocarcinoma of the lung \((n = 2)\), squamous lung cell cancer \((n = 1)\), and large-cell lung carcinoma \((n = 1)\)], and anal cancer \((n = 5)\). Hematological malignancies were acute lymphoblastic leukemia \((n = 5)\), acute myeloid leukemia \((n = 4)\), chronic myeloid leukemia \((n = 1)\), multiple myeloma \((n = 1)\), myeloproliferative and myelodysplastic syndrome \((n = 1)\), and Hodgkin or non-Hodgkin lymphoma \((n = 8)\).

Chemotherapeutic Agents and Associated Therapy (Radiotherapy and BMT)

From all identified cases in the French PH network and systematic review \((n = 37)\), exposure to the following

![Figure 6](https://example.com/figure6.png)

**Figure 6** Cyclophosphamide (CP)—induced pulmonary hypertension is partially prevented by amifostine pretreatment, but not by mesna. Amifostine was given i.p. at 200 mg/kg 30 minutes before 200 mg/kg per week for 2 weeks of CP. Mesna dose was given as i.p. injections in a dosage equal to 20% of CP dose at time of administration, 4 hours after each CP dose, and 8 hours after each CP dose. Animals were sacrificed 4 weeks after the second CP injection. Hemodynamic parameters, right heart hypertrophy, and survival were evaluated. **A:** Fulton index [right ventricle (RV)/left ventricle (LV) + septum (S) weight ratio]. **B:** Mean pulmonary artery pressure (mPAP; in mmHg). **C:** Cardiac output (CO; in mL/minute). **D:** Total pulmonary resistances (TPRs; in mmHg/mL per minute). **E:** Survival curves for CP, CP + Mesna, and CP + Amifostine groups. *\( P < 0.05 \), **\( P < 0.01 \), and ****\( P < 0.0001 \).
chemotherapeutic classes occurred before the development of PVOD (Table 3): alkylating agents or alkylating-like agents in 83.8% of cases, antimetabolites in 40.5% of cases, plant alkaloid and naturally occurring molecules in 45.9% of cases, and cytotoxic antibiotic and related molecules in 43.2% of cases. Alkylating or alkylating-like agents were mostly represented by CP (43.2%), mitomycin (24.3%), and cisplatin (21.6%). In addition, 54% of all patients received additional radiotherapy, including total body irradiation in seven patients (18.9%), thoracic radiotherapy in two patients (5.4%), and local radiotherapy in 11 patients (29.7%). Allogeneic stem cell transplantation has been proposed in eight patients (21.6%), and autologous stem cell rescue was reported in five patients (13.5%).

These results suggested that exposure to alkylating agents, in particular CP, was the most frequent risk factor observed in chemotherapy-induced PVOD. To confirm this observation, we conducted experiments using CP in three different animal models (mouse, rat, and rabbit).

CP Induces PH in Mouse

When mice were injected with a single dose of 350 mg/kg CP, they developed PH without an apparent sex difference. This was attested 4 weeks later by a significant increase in right ventricular systolic pressure and by compensatory RVH quantified by the Fulton index [RV/(LV + S)] (Figure 2, A–D). This macroscopic heart effect was specific to the RV because only the ratio of RV/body weight increased after CP exposure, but not the ratio of LV/body weight. Histologically, we observed septal thickening and accumulation of large foamy intra-alveolar macrophages (Figure 2, E and F).

Pulmonary Vascular Remodeling, Endothelial Dysfunction, and Effects of Cytoprotective Agents on CP-Induced PH in Rat

In rats, there was a sex difference in response to a single injection of 350 mg/kg CP. Females developed severe and homogeneous PH after 4 weeks of treatment characterized by marked RVH (Supplemental Figure S1), whereas males developed heterogeneous PH ranging from nonaffected to severely affected animals. Histologically, affected animals had distal muscularization of normally nonmuscularized microvessels (arterioles and venules; <50 μm) (Figure 3, B, C, E, and F). This was associated with medial hypertrophy of more proximal muscularized pulmonary arteries (Figure 3, A–D). After this initial study, we, therefore, chose females to analyze the kinetics of PH development after a single 350 mg/kg CP exposure over a 4-week period.
We found that mean PA pressure and Fulton index were significantly increased 3 weeks after CP exposure and worsened further at 4 weeks (Supplemental Figure S2, A and B). The appearance of symptomatic PH at 3 weeks was preceded by an early and dramatic decrease in low-resistance NM distal microvessels at 1 week and, at the same time, an opposite large increase in partially muscularized distal microvessels. Subsequently, vascular remodeling continued to progress as the percentage of fully muscularized distal microvessels became significantly increased at 2 weeks and continued to increase at 3 and 4 weeks (Supplemental Figure S2, C–F). Early pulmonary vascular remodeling occurring at 1 and 2 weeks after CP exposure coincided with depletion of T- and B-lymphocyte populations in the peripheral blood (Supplemental Figure S3).

To reduce whole body toxicity of a single high dose of 350 mg/kg CP, we performed a dose-response study using fractionated doses in female rats at 100, 150, 200, and 250 mg/kg per week for two doses, with sacrifice at 4 weeks after the second injection. No rat survived the highest dose to 4 weeks. All other doses induced PH with significant increase in Fulton index and mean PA pressure. A dose-response relationship to CP was observed, as evidenced by higher CO, lower total pulmonary vascular resistances (Figure 4), and lower percentage of occluded distal microvessels (>50 μm) (Figure 5) in the lower-dose groups. Serum vascular endothelial growth factor levels were increased across all dose ranges, endothelial dysfunction markers soluble E-selectin and vWF were elevated for the two highest doses, and finally, heart failure biomarker, B-type natriuretic peptide, was increased only for the 200 mg/kg per week for 2 weeks (Supplemental Figure S4, A–D). No significant increases in other serum markers of endothelial dysfunction and inflammation (sICAM, monococyte chemoattractant protein-1, troponins T and I, tissue inhibitor of matrix metalloproteinases type I, plasminogen activator inhibitor 1, myeloperoxidase, IL-6, and tumor necrosis factor α) were detected. Last, we measured the level of serotonin (5-HT) in the lungs of control and CP-exposed rats by LC-MS/MS, given the recognized role of 5-HT in human PH pathogenesis.29 We found that only the two highest doses of CP led to significant pathological pulmonary 5-HT accumulation (Supplemental Figure S4E). Hence, in the next step of the study, we used CP at 200 mg/kg per week for 2 weeks.

Figure 9  Cyclophosphamide (CP) induces pulmonary hypertension in rabbits with signs of vein-related vasculopathy. A–P: Representative images of the pulmonary precapillary and post-capillary and capillary vessels of nonexposed (A, B, E, F, I, J, M, and N) and CP-exposed (C, D, G, H, K, L, O, and P) rabbits. C and D: CP induces medial hypertrophy of muscular pulmonary arteries (PAs) compared to the thin control PAs (A and B). CP induces pulmonary vein (PV) wall thickening and PV adventitial and transmural inflammatory infiltration and fibrosis (G and H) compared to control PVs (E and F). CP induces muscularization of distal microvessels with foci of pulmonary congestion (K and L) compared to control microvessels and parenchyma (I and J). CP induces foci of septal hyperplasia and alveolar inflammation (O and P) compared to control alveoli and alveolar walls (M and N). Hematein-eosin-safran stain was used. Boxed areas are shown at higher magnification in the panels to their right. Scale bars: 100 μm (A–E, G, I, and K); 50 μm (F, H, J, and L–P).
weeks as a model of severe CP-induced PH with right ventricular failure. It has recently been shown that heritable pulmonary arterial hypertension can be related to missense mutations in KCNK3 (the gene encoding KCNK3), resulting in loss of function, and the reduction in the potassium channel current.\textsuperscript{30} Accordingly, CP-induced pulmonary vascular dysfunction was associated with decreased expression of KCNK3 protein in the lungs (Supplemental Figure S5).

Mesna and amifostine are cytoprotective agents given in combination with chemotherapy to reduce normal tissue toxicity. Therefore, we assessed the potential prevention of CP-induced PH by these two cytoprotective agents\textsuperscript{31,32} using standard administration protocols. In the prevention study, amifostine, but not mesna, ameliorated CP-induced PH, with a notable improvement in survival and pulmonary hemodynamics (increase in CO with decrease in total peripheral vascular resistance) in CP + amifostin–treated rats (Figure 6).

The decrease of total pulmonary resistance in CP + amifostin–treated rats was linked to a decrease in resistive fully muscularized distal microvessels, and an increase in low-resistance NM-distal microvessels (Figure 7, A–C). The percentage of occluded distal microvessels was significantly lowered in both amifostine- and mesna-treated groups (Figure 7D). Inflammation, quantified by the total protein level of the pan-leukocyte marker CD45, was decreased in both cytoprotective strategies (Figure 7E). Both mesna and amifostine significantly decreased serum B-type natriuretic peptide levels, but had minor effects on vWF and soluble E-selectin levels (Supplemental Figure S6, A–C). However, only amifostine decreased the pathological pulmonary accumulation of 5-HT in CP-exposed animals (Supplemental Figure S6D). The lung protein level of KCNK3 in CP-exposed rats was not normalized by either amifostine or mesna (Supplemental Figure S7).

Finally, we assessed vascular remodeling after long-term exposure to CP. It has been shown in rat models of severe PH\textsuperscript{33} that pathognomonic angio proliferative lesions of human PAH (plexiform lesions) can only be obtained after 13 to 15 weeks of disease induction. Therefore, a lower dose of CP (100 mg/kg) was used to allow repeated injections of CP without premature death due to early heart failure. However, after 13 weeks of repeated low-dose CP exposure, we only observed intimal thickening and sporadic presence of recanalized small pulmonary vessels, but there was an absence of plexiform lesions (Figure 8).

**Pathological Assessment of CP-Induced PH in Rabbit**

On the basis of results obtained in rat, we challenged female rabbits. The maximal tolerated CP dose was 100 mg/kg per injection. Three injections of CP resulted in moderate PH, confirmed by right heart hypertrophy measured by Fulton index (Supplemental Figure S8). Histologically, we found medial hypertrophy of muscular PA, neomuscularization of distal microvessels, and congestion and hyperplasia of septa. More important, there were significant thickening and adventitial fibrosis of pulmonary veins in association with a pronounced vasculitis (Figure 9). These pathological changes are consistent with venular remodeling resembling venoocclusive disease. Pulmonary vascular eosinophilic inflammation was prominent in CP-exposed rabbits, leading to severe vascular remodeling and intimal thickening (Figure 10).

CP-induced PH in rabbit was associated with a significant increase in plasma endothelin-1 levels, a potent vasoconstrictor involved in human PAH (Supplemental Figure S9).

**Discussion**

Our systematic review and experience of the French PH network suggest that alkylating and alkylating-like agents represent a risk factor for the development of PVOD. In experimental models, CP exposure induced PH in three different animal models: mouse, rat, and rabbit. In rats, the severity of PH and vascular remodeling was sex dependent (females were more susceptible than males), time dependent, and dose dependent. We also demonstrate in rats that amifostine pretreatment improved survival and ameliorated PH severity after CP exposure. Mesna did not appear to share the
same cytoprotective effects on the pulmonary vasculature. Rabbits exposed to CP displayed features of congestive lungs and venular involvement, mimicking human PVOD. In mice, it has been shown previously that a single injection of CP can produce pulmonary toxicity characterized by accumulation of intra-alveolar macrophages and diffuse progressive interstitial fibrosis. We demonstrate, for the first time, that CP can also induce pulmonary vascular remodeling and PH in mice.

The similarity of endothelial cell response to different bifunctional alkylating agents suggests that DNA cross-linking may inhibit cell proliferation and thereby limit the repair capacity of endothelial monolayers. This may contribute to the progressive pulmonary vascular injury that occurs after administration of certain DNA cross-linking agents in vivo. The pattern of pulmonary endothelial cell injury induced by chemotherapy agents is reminiscent of that seen after treatment with another well-known bifunctional alkylating agent, monocrotaline, pyrrole (the active metabolite of monocrotaline), which is commonly used to trigger experimental PH in rats. CP is a common component of multidrug regimens, with a high potential for pulmonary toxicity, and has been reported to cause acute and chronic pulmonary injury in both humans and animals. The activities of the enzymes involved in the metabolism of CP show significant tissue selectivity, and the lack of detoxifying enzymes, such as aldehyde oxidase and aldehyde dehydrogenase, in the lungs accounts for selective CP toxicity in lung tissue. Furthermore, it has been demonstrated that endothelial cells are more susceptible to the effects of CP than other cell types. Previous in vitro studies with CP, busulfan, azathioprine, monocrotaline, and dacarbazine suggest that these drugs can cause hepatic veno-occlusive disease by targeting sinusoidal endothelial cells via glutathione (GSH) depletion. Rats exposed to CP show reduced pulmonary GSH content, glucose-6-phosphate dehydrogenase, GSH reductase, GSH peroxidase, and superoxide dismutase activities. Therefore, one mechanism of pulmonary toxicity of CP could be mediated by oxidative damage.

The present article represents the largest systematic review of the literature regarding the possible association between PVOD and chemotherapeutic agents. Most of the patients were treated by different chemotherapeutic agents and, therefore, a clear relationship between specific drug use and PVOD is difficult to establish. Nevertheless, on the basis of observations from the literature review, alkylating agents are often identified to be associated with the development of PVOD. The identification of alkylating agents could also be linked to the fact that these drugs are frequently used in the treatment of solid or hematological malignancies. However, the frequent identification of alkylating agents, mainly represented by CP and mitomycin, suggests a likely relationship between PVOD and exposure to these agents. The analysis of case reports in the literature was based on clinical, functional, histopathological, and hemodynamic parameters. The diagnosis of PAH and PVOD is sometimes difficult to distinguish because of similar clinical presentation and overlapping histological changes. However, all reported cases were reviewed by experts of different specialties, and histological confirmation was present in >50% of cases.

In our experimental models of CP exposure, the development of PH was associated with pulmonary venous remodeling. Although lung injury leading to interstitial fibrosis is a well-documented potential complication of various chemotherapeutic agents, the association between vascular injury and CP represents a novel finding. The pathophysiological features of PVOD in the setting of chemotherapy and the mechanisms leading to pulmonary venous remodeling and capillary proliferation are largely unknown. Recently, it has been demonstrated that a heritable form of PVOD is due to biallelic mutation of the EIF2AK4 gene. EIF2AK4 gene codes for GCN2, a serine-threonine kinase that can induce changes in gene expression in response to amino acid deprivation. The role and expression of GCN2 in the pulmonary vasculature are largely unknown; however, a decrease of GCN2 activity may lead to an increase in vulnerability to oxidative stress and an increase in inflammation. EIF2AK4 (T−/−) knockout mice have been shown to display increased susceptibility to both acute or chronic liver damage induced by carbon tetrachloride, which is accompanied by increased necrosis and greater inflammatory infiltrates compared to wild-type mice. Interestingly, we also noted a pronounced vasculitis of small pulmonary veins in CP-exposed animals. Because only a minority of patients treated with alkylating agents will develop PVOD, further studies on genetic susceptibility and the role of GCN2 in human chemotherapy–induced PVOD are required.

It is paradoxical that CP has been used in clinical practice, with therapeutic success for PAH in the setting of inflammatory conditions, such as systemic lupus erythematosus and mixed connective tissue disease. We can speculate that CP might reverse PAH when associated with inflammatory conditions, but it may induce PVOD in subjects with underlying susceptibility. As an analogy, such paradoxical effects have also been observed with dasatinib, a dual Src/Abl kinase inhibitor, which is able to reverse experimental PH but induces PAH in humans.

In conclusion, we demonstrate a plausible cause-effect relationship between PVOD and chemotherapeutic agents. From our exhaustive review of the literature and French PH network, alkylating or alkylating-like agents are particularly implicated in chemotherapy-induced PH. In this context, we evaluated different experimental models of PH and demonstrated that CP, an alkylating agent, can induce PH with pulmonary venous involvement. Clinicians should be aware of this uncommon, but severe, pulmonary vascular complication of alkylating agents.

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Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.amjpath.2014.10.021.

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