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Relative Contributions of Mouse Liver Subcellular Fractions to the Bioactivation of Mitomycin C at Various pH Levels

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ABSTRACT. Mitomycin C (MMC) is a clinically active anticancer drug that requires reductive activation to exert its toxicity. The enzymes currently recognized as capable of activating MMC cannot account for all of the toxicity of the drug. These studies were conducted to identify and compare the subcellular compartments where MMC reduction can take place under different physiological conditions. Subcellular fractionation of mouse liver was achieved using differential centrifugation and isopycnic equilibrium gradient centrifugation. Nuclear, mitochondrial, microsomal, lysosomal, peroxisomal, and cytosolic fractions were assayed for their ability to reductively activate MMC at pH 6.0 and 7.4. MMC reductive activation was determined by its ability to generate reactive oxygen species. The results of these studies showed that MMC reductive activation by the various fractions was pH dependent. At pH 7.4, the microsomal fraction accounted for approximately 78% of the total MMC reductive activation. The peroxisomal fraction accounted for 12% and the nuclear and lysosomal fractions each accounted for 5% of the total reductive activation. At pH 6.0, the microsomes accounted for 51% and the peroxisomes for 34% of the total reductive activation. The mitochondrial fraction, which did not reductively activate MMC at pH 7.4, accounted for 9% of the total activation at pH 6.0. These results suggested that peroxisomes may be important in MMC activation at either pH and that at pH 6.0 the mitochondrial fraction may also be important for MMC reductive activation. BIOCHEM PHARMACOL **58**;10:1609–1614, 1999.

KEY WORDS. bioactivation; subcellular fractions; mitomycin C; peroxisome; lysosome; mitochondria

MMC† is a clinically used chemotherapeutic agent that requires biological activation to exert its toxicity [1, 2]. The complex structure of the MMC molecule allows it to form multiple metabolites capable of alkylation and/or redox cycling depending upon the conditions surrounding the molecule at the time of reductive activation and the enzyme involved in the activation. Some enzymes, such as DT-diaphorase [3] and xanthine dehydrogenase [4], can activate MMC by a two-electron reduction. Other enzymes, such as NADPH-cytochrome P450 reductase [5], xanthine oxidase [5], and cytochrome b_5 reductase [6], reduce MMC by one electron. Metabolite formation and, subsequently, toxicity can be different depending upon whether the reductive activation is by a one- or two-electron reducing enzyme.

Other important considerations in MMC activation include oxygen concentration and pH. MMC has been shown to exert preferential toxicity to hypoxic cells in a number of cell lines [7, 8]. This differential toxicity, however, is not observed in all cell lines [8] and presumably is due to differences in enzyme activities. A number of studies with various cell lines have used inhibitors of different enzymes to assess the role of these enzymes in activating MMC to a toxic metabolite [8–10]. These studies have shown that some enzymes, such as DT-diaphorase, appear to be very important in some cell lines under particular conditions. However, the use of enzyme inhibitors has not been able to account for all of the cytotoxicity observed with the drug. Therefore, it is clear that other enzyme systems, as yet unidentified, are involved in the activation of MMC to toxic metabolites. A recent study suggested that a mitochondrial reductase found in the murine adenocarcinomas MAC 16 and MAC 26 can reductively activate MMC to form toxic metabolites [11]. The enzyme responsible for the activation, however, has not been identified. Earlier studies by Schwartz [12] and later by Kennedy *et al.* [13] identified the microsomal fractions of rat and mouse livers, respectively, as being capable of metabolizing and activating MMC under anaerobic conditions. Kennedy *et al.* [13] further demonstrated that mouse liver nuclei contain enzymes capable of activating MMC anaerobically, although to a much lesser degree than mouse liver microsomes. Studies that identify other

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† *Abbreviations*: MMC, mitomycin C; and mt-DNA, mitochondrial DNA.

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subcellular fractions capable of MMC reductive activation have not been conducted.

The cellular pH at the time of MMC administration can greatly influence the activation of this drug. At pH levels of less than 4.5, MMC has been shown to be activated at its aziridine moiety to alkylate DNA at the N7 position of guanine [14]. Although this particular activation may not be biologically relevant, pH has been shown to affect the catalytic potential of enzymes that activate MMC. It has been hypothesized that cells located distal to the blood supply of a solid tumor not only have a reduced oxygen level but also have a more acidic pH than their more oxygenated counterparts located proximal to the blood supply [15]. DT-diaphorase [3], xanthine dehydrogenase [4], and cytochrome b_5 reductase [6] activation of MMC all have been shown to be affected by pH within the ranges estimated to occur within solid tumors.

It is evident that other, perhaps important, enzymes capable of biologically activating MMC remain to be identified. This study was designed to determine the relative ability of various subcellular fractions, obtained through differential centrifugation of mouse liver homogenates, to reductively activate MMC under aerobic conditions. MMC reductive activation in these studies was determined by measuring MMC-induced oxygen uptake. This is primarily a measure of the one-electron reduction of MMC by these fractions. Under hypoxic conditions, this one-electron reduction would generate an MMC semiquinone metabolite capable of alkylation reactions. The identification of subcellular fractions previously unknown to be able to reduce MMC could provide new directions in the search for MMC-activating enzymes and new insights into its mechanism of action.

MATERIALS AND METHODS

MMC was purchased from ICN. NADPH, NADH, *o*-nitrophenyl acetate, uric acid, lithium carbonate, cytochrome *c*, and *p*-nitrophenol-acetylglucosaminide were purchased from the Sigma Chemical Co. All other reagents were of analytical grade.

Cellular Fractionation

Homogenates were prepared from the livers of Balb/c mice deprived of food for 24 hr. The homogenates were prepared in a medium containing 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA, and 0.1% (v/v) ethanol at a final pH of 7.4. The homogenate was diluted with sucrose buffer to a concentration of 7–10 mL/g liver. Subcellular fractions were prepared according to the differential centrifugation method of de Duve [16]. Briefly, the homogenate was centrifuged for 10 min at 600 *g* to pellet the nuclear fraction. The resulting supernatant was centrifuged for 10 min at 3,000 *g* to pellet the heavy mitochondrial fraction, and the resulting supernatant then was centrifuged at 25,000 *g* for 10 min to obtain a light mitochondrial pellet. The supernatant was removed from the light mitochondrial pellet and centrifuged at 100,000 *g* for 30 min to obtain the microsomal fraction (pellet). The supernatant from the 100,000 *g* spin constituted the cytosolic fraction. Lysosomes and peroxisomes were isolated subsequently from the light mitochondrial pellet using isopycnic equilibrium gradient centrifugation. The light mitochondrial pellet was resuspended in sucrose buffer and divided into two fractions. One fraction was used for lysosomal isolation and the other for peroxisomal isolation. Peroxisomes were separated from the light mitochondrial fraction using a linear Nycodenz gradient (20–50%, w/v) following centrifugation for 2 hr at 100,000 *g* as described by Wilcke and Alexson [17]. Lysosomes were separated on a linear gradient by a method similar to the one described by Dobrota [18]. In this method, Dobrota used the density gradient compound metrizamide. We used the same procedures but substituted Nycodenz for metrizamide to form the gradient. Nycodenz is a more stable and inert compound that prevents the interferences seen in some chemical and enzymatic assays with metrizamide [19]. All pelleted fractions were resuspended in 50 mM potassium buffer (pH 7.4 or 6.0), except for the peroxisomes and lysosomes, which remained in the Nycodenz solution.

Marker Enzyme Analysis

The enzymatic activity of specific marker enzymes was determined for each of the isolated fractions to determine the purity of the various fractions. Enzymatic analysis using standard methods was conducted for uricase [20], a marker enzyme for peroxisomes; β-*N*-acetylhexosaminidase [21], a marker enzyme for lysosomes; esterase [22], a marker enzyme for microsomes; and succinate cytochrome *c* reductase (succinate dehydrogenase) [23], a marker enzyme for mitochondria. The purity of each of the fractions was calculated by the method described by Wattiaux *et al.* [24] using marker enzyme activities.

Oxygen Consumption Analysis

Subcellular fraction-activated MMC-induced oxygen consumption studies were conducted using a YSI Biological Oxygen Monitor equiped with a Clarke electrode. Assay mixtures contained 50 mM potassium phosphate buffer (pH 7.4 or 6.0), 6 mM NADH, 6 mN NADPH, 300 μM MMC, and crude sample to a final volume of 1.34 mL. The buffer and sample were added to the chamber and allowed to equilibrate to 25°. NADH and NADPH were added, and a baseline rate of oxygen consumption was recorded. Both cofactors were added to the assay mixture to provide both forms of reduction potential. This allows one to account for enzymes requiring either one of the cofactors. Once the baseline rate was established, MMC was added, and the new rate of oxygen consumption was determined. The MMC-induced rate of oxygen consumption was obtained

TABLE 1. Specific activities of marker enzymes in subcellular fractions from mouse liver

Subcellular fraction	Uricase (U/mg protein)	Hexosaminidase (μmol/min/mg protein)	Esterase (μmol/min/mg protein)	Succinate dehydrogenase (μmol/min/mg protein)	Fraction purity* (%)
Nuclear	0	19.41	86.2	4.7	
Mitochondrial	0	33.34	118.6	41.1	>93
Microsomal	0	13.83	270.2	0.77	>95
Cytosolic	0	2.91	3.10	0.4	
Peroxisomal	0.014	0	7.2	1.4	>94
Lysosomal	0	57.92	14.2	17.0	>96

*The purity of the mitochondrial, microsomal, peroxisomal, and lysosomal fractions was determined by the activity of the marker enxymes shown in this tanble, by the method described by Wattiaux *et al.* [24].

by subtracting the MMC-induced rate from the baseline rate.

RESULTS

Subcellular fractionation of the mouse liver homogenate by differential centrifugation followed by isopycnic equilibrium gradient centrifugation in a linear Nycodenz gradient provided us with six subcellular fractions, which were tested for MMC reductive bioactivation potential. These subcellular fractions (nuclear, mitochondrial, microsomal, lysosomal, peroxisomal, and cytosolic) were assayed for marker enzymes in order to assess the degree of purity. Succinate dehydrogenase was used as the marker enzyme for mitochondria, esterase was used as the marker enzyme for microsomes, uricase was used as the marker enzyme for peroxisomes, and β-*N*-acetylhexosaminidase was used as the marker enzyme for lysosomes. The activities of the various marker enzymes in each of the subcellular fractions are shown in Table 1. The purity of the fractions was determined from the activity of the marker enzymes in each fraction as described by Wattiaux *et al.* [24]. The results also are shown in Table 1. These results demonstrate that a good separation of the various organelles was obtained by this method, with less than 7% contaminating protein in any subcellular fraction.

MMC-induced oxygen consumption for each of the various subcellular fractions at pH 7.4 and 6.0 is shown in Table 2. MMC-induced oxygen consumption measures the ability of the drug to generate oxygen radicals following reductive activation. A portion of the two-electron reduced hydroquinones may react with and consume molecular oxygen directly or indirectly through a disproportionation reaction between the fully reduced hydroquinone and the oxidized quinone to form the semiquinone, which can then react with oxygen. However, these results primarily reflect the reaction of the one-electron reduction of MMC to the semiquinone form of the drug, which under aerobic conditions can react subsequently with molecular oxygen to form superoxide, thereby consuming molecular oxygen. Under hypoxic conditions the MMC semiquinone metabolite is more stable and, hence, is capable of alkylating important cellular macromolecules. The results showed that MMC reductive activation by the various fractions was pH dependent, with the nuclear, microsomal, and lysosomal fractions showing a decrease in MMC activation as the pH was decreased to 6.0, and the mitochondrial and peroxisomal fractions showing an increase in activity as the pH was decreased to 6.0. Table 3 shows the relative contribution of each of the subcellular fractions to total MMC activation based upon the oxygen consumption data presented in Table 2 as well as the percentage of cellular protein represented by each of the subcellular fractions, as previously determined by Leighton *et al.* [25] for rat liver. These

TABLE 2. MMC-induced stimulation of oxygen consumption at pH 7.4 6.0*

Subcellular	O_2 consumed (nmol/min/mg protein) pH 7.4	pH 6.0
Nuclear	9.05 ± 6.0	1.68 ± 0.23
Mitochondrial	0	4.28 ± 1.57
Microsomal	47.2 ± 11.0	23.0 ± 5.94
Cytosolic	0	0
Lysosomal	32.3 ± 19.6	21.25 ± 0.02
Peroxisomal	60.2 ± 21.9	128.7 ± 0.75

*Values represent the means ± SD from at least six separate experiments.

TABLE 3. Relative contribution of each subcellular fraction to total MMC activation

Subcellular fraction	% Cellular protein*	% MMC activation† pH 7.4	pH 6.0
Nuclear	7.5	5	1
Mitchondrial	20.2	0	9
Microsomal	21.5	78	51
Cytosolic		0	0
Lysosomal	2.0	5	5
Peroxisomal	2.5	12	34

*The percentage of cellular protein represented by each of the subcellular fractions was obtained from the data published by Leighton *et al.* for rat liver [25].

†The percentage of total cellular MMC activation represented by each of the subcellular fractions was obtained in the following manner. The MMC-induced O_2 consumption values (nmol O_2 consumed/min/mg protein) obtained for each subcellular fraction at each pH was multiplied by the percent cellular protein represented by each subcellular fraction. For each pH, a total value was determined by adding the values obtained for each of the subcellular fractions. The value for each subcellular fraction was then divided by the total value obtained for all the fractions (for a specific pH) and multiplied by 100 to arrive at the relative percent of contribution for each subcellular fraction.

results demonstrated that pH has an important effect on the relative ability of the subcellular fractions to reductively activate MMC. At pH 7.4, activation by the microsomal fraction accounted for more than three-quarters of the total reductive activation. Peroxisomal activation accounted for approximately 12% of the total activation, and lysosomal and nuclear fraction activation accounted for 5% each. The mitochondrial and cytosolic fractions had no ability to reductively activate MMC at pH 7.4. When the pH was reduced to 6.0, the total subcellular MMC activating potential was reduced by 25%. The relative contribution of each of the fractions also was affected in that microsomes contributed only 51% of the total reductive activation at this pH, and peroxisomes contributed about 34% of the total activation. It is also interesting that at pH 6.0 the mitochondria were capable of reductively activating MMC to a significant degree. Lysosomes still accounted for approximately 5% of the total activation, whereas the nuclear fraction accounted for only 1%, and the cytosolic fraction did not appear to reductively activate MMC at all.

The pH dependence for the reductive activation of MMC by the mitochondrial fraction was explored further by determining its ability to activate MMC-induced oxygen consumption at the intermediate pH of 6.7. The results of these studies are shown in Fig. 1A. Figure 1B shows the results of linear regression conducted on these points; a linear relation appeared to exist between mitochondrial activation of MMC-induced oxygen consumption and pH.

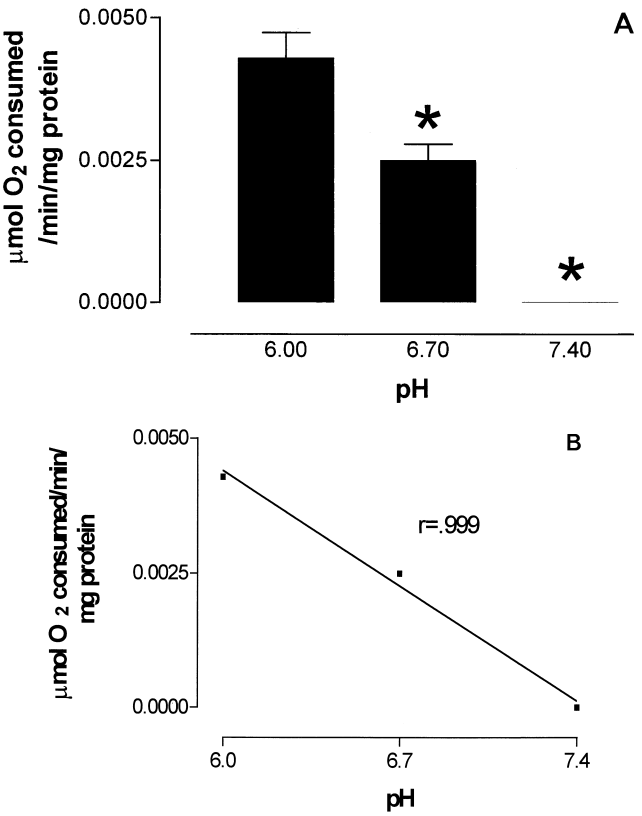

FIG. 1. MMC-induced oxygen consumption at various pH levels following reductive activation by the mitochondrial fraction. (A) Oxygen consumption data; values are means ± SD (N = 6). Key: (*) values that are statistically different from each other by ANOVA ($P < 0.05$). (B) Linear regression analysis of the data from panel A.

DISCUSSION

The successful use of bioreductive chemotherapeutic agents for the eradication of malignant solid tumors depends upon a number of factors. Included in these factors is an understanding of the enzymes capable of activating the bioreductive compound to form its active metabolites. Once this is understood, an enzymatic profile for tumors of individual patients can be obtained, and a therapeutic treatment can be designed that will deliver the appropriate bioreductive agents according to the enzymatic profile. This type of therapeutic approach should greatly improve the success of these agents in the clinic.

MMC, the prototype bioreductive chemotherapeutic agent, has been studied extensively. Thus far, five enzymes have been identified that are capable of activating MMC to form reactive metabolites. NADPH-cytochrome P450 reductase [5], cytochrome b_5 reductase [6], and xanthine oxidase [5] all have been shown to activate MMC via a one-electron reduction. DT-diaphorase [3] and xanthine dehydrogenase [4] have been shown to activate MMC via a two-electron reduction. A mitochondrial reductase capable of activating MMC via one-electron reduction has also been characterized but not identified as yet [11]. Studies that have utilized enzyme inhibitors to assess the role of the known enzymes in MMC activation have not been able to inhibit the activity of MMC sufficiently to account for its total activation by these enzymes [8]. It is clear from these studies that other, as yet unidentified, enzymes exist that can activate MMC to form toxic metabolites.

Early studies were directed toward understanding the metabolism and activation of MMC by microsomes [13, 26–28] or nuclei [13] isolated from rat or mouse livers. A few studies also have looked at MMC activation by microsomes and nuclei isolated from various tumor cell lines [8, 29]. Spanswick *et al.* [11] characterized the activation of MMC in the cytosol, microsomes, and mitochondria of two murine adenocarcinomas. The studies presented here are the first to assess the activation of MMC by subcellular fractions following extensive cellular subfractionation. We fractionated mouse liver homogenates into six subcellular fractions (nuclear, mitochondrial, microsomal, peroxisomal, lysosomal, and cytosolic) and assayed them for their ability to activate MMC at pH 6.0 and 7.4.

The results of these studies showed that the relative contributions of the various fractions to activate MMC-induced oxygen consumption were different at various pH levels. The ability of both the peroxisomal and lysosomal fractions to reductively activate MMC and the lack of activation by the resultant cytosolic fraction is quite interesting. This suggests that MMC activation previously ascribed to the cytosolic fraction in other studies may actually

be due to peroxisome- and/or lysosome-induced activation. Both peroxisomes and lysosomes have NADH and NADPH-cytochrome P450 reductase activity. In rat liver, peroxisomes and lysosomes have approximately 21 and 50% of the NADH-cytochrome *c* reductase specific activity found in microsomes, respectively [30]. Rat liver peroxisomes contain 14% and lysosomes contain 27% of the specific activity of NADPH-cytochrome *c* reductase found in corresponding microsomes [30]. Rat liver peroxisomes also have 24% of the specific activity of NADH-cytochrome b_5 reductase found in corresponding microsomes [30]. Xanthine oxidase activity also has been reported in peroxisomes [31]. Neither NADH-cytochrome b_5 reductase nor xanthine oxidase activity has been reported in lysosomes. It is clear from these studies that both peroxisomes and lysosomes contain enzymes capable of activating MMC and that MMC is activated by these organelles. At pH 6.0, the contribution of these organelles to the reductive activation of MMC became highly important, with peroxisomes contributing approximately 34% of the total activation and lysosomes approximately 5% of the total MMC activation. Peroxisomal activation at pH 6.0 was exceeded only by microsomal activation. MMC activation within the mitochondrial fraction also may prove to be an important aspect of toxicity.

Recent studies from our laboratory demonstrated that MMC administered to EMT6 mouse mammary carcinoma cells in cell culture results in a concentration-dependent conformational change in their mt-DNA [32]. This strongly suggested that MMC is capable of interacting with the mt-DNA in such a way as to damage it. We have also demonstrated that MMC-treated Balb/c mice not only have dysfunctional mitochondria [32] but also have decreased tissue ATP levels [33], further implicating a mitochondrial mechanism of toxicity for MMC. Early work by Doroshow [34], however, showed that MMC does not undergo redox cycling with the mitochondrial respiratory chain from rat heart. These results suggest that MMC activation occurs either outside of the mitochondria or by enzymes other than the respiratory chain enzymes. A number of possibilities exist. As stated previously, Spanswick *et al.* [11] have characterized a mitochondrial reductase from tumor cells capable of activating MMC. Those studies were conducted under both aerobic and hypoxic conditions at pH 7.4, and it is unclear how pH might have affected the rate of activation by this reductase. The data suggested to the authors that the MMC activation was by a one-electron reduction. The formation of metabolites was inhibited completely by oxygen, which also suggests MMC redox cycling under aerobic conditions. In our studies, we did not observe any oxygen consumption at pH 7.4 in our mitochondrial fraction. Either our assay for oxygen consumption is not sensitive enough to detect the level of activation and subsequent oxygen consumption, or perhaps these tumor cell mitochondria have enzymes not present or with different pH optima than those in mouse liver mitochondria. As our oxygen consumption determinations can detect oxygen consumption rates in nanomoles of O_2 consumed per minute, it appears that this is a sensitive enough assay to detect the redox cycling of MMC by this enzyme, and, therefore, supports the latter hypothesis. If correct, this difference could be exploited in developing drugs directed for activation by this enzyme. Hodnick and Sartorelli [6] have shown that cytochrome b_5 reductase can activate MMC via a one-electron reduction to alkylate 4-(*p*-nitrobenzyl)pyridine and consume oxygen. The activation of MMC by cytochrome b_5 reductase in this system is pH dependent, with greater activity seen at pH 6.6 than at 7.4. Cytochrome b_5 reductase has been shown not only to occur in the microsomes but also to be present in peroxisomes as well as the outer mitochondrial membrane. The role of cytochrome b_5 reductase-induced activation in our mitochondrial fraction is unclear at this time, as the comparison between our two studies is mixed. Hodnick and Sartorelli [6] observed significant MMC activation at pH 7.4, whereas we did not. They also observed an increase in activation as the pH was decreased to 6.6. We observed a linear increase in MMC activation in the mitochondrial fraction as the pH was decreased from 7.4 to 6.0. A soluble form of cytochrome b_5 reductase, purified from rabbit erythrocytes, was used in the study of Hodnick and Sartorelli [6]. This form could be different from the mouse liver outer mitochondrial membrane form that would have been present in our fraction. Activation of MMC by enzymes located on the outer mitochondrial membrane still would require the activated metabolite to be transported across the inner mitochondrial membrane to damage the mt-DNA. Therefore, it appears to us that it is more likely that enzymes in the mitochondrial matrix may be required for activation of MMC to interact with mt-DNA. This could be the mitochondrial reductase characterized by Spanswick *et al.* [11], mitochondrial DT-diaphorase, or some as yet unidentified enzyme. However, this does not rule out the possibility that MMC activated by outer mitochondrial membrane cytochrome b_5 reductase could inhibit the mitochondrial respiratory chain. Previous studies, such as the one conducted by Doroshow [34] that showed no interaction between MMC and the mitochondrial respiratory chain, used isolated mitochondria, which would lack the outer mitochondrial membrane and thus would not contain the cytochrome b_5 reductase enzyme. Another issue worth consideration in assessing potential mitochondrial-enzyme-induced activation of MMC is the pH of the mitochondria. During active respiration, the pH in the mitochondrial intermembrane space becomes more acidic as protons are pumped out of the mitochondrial matrix into this space between the inner and outer membranes. This reduced pH could affect enzyme-induced MMC activation in this compartment.

These studies have provided important new data relevant to the activation of MMC in various mouse liver subcellular fractions at different pH levels. The significance of these results is that they identify subcellular fractions capable of MMC activation, which were previously unknown. They also provide insights into the relative MMC activation by

the various subcellular fractions at different pH levels. These results provide additional understanding of the cellular activation of MMC.

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